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PROCEEDINGS OF

THE ROYAL SOCIETY.

SECTION B.—BIOLOGICAL SCIENCES.

Oogenesis in Oniscus Asellus.

By Miss S. D. KING, B.A., Sc.B., Ph.D., Assistant to the Professor of Zoology and Comparative Anatomy, Trinity College, Dublin.

(Communicated by Prof. E. S. Goodrich, F.R.S.—Received January 22,—Revised March 24, 1926.)

[PLATES 1 AND 2.]

The behaviour of the cytoplasmic inclusions during oogenesis has been investigated in many animals by different observers, and the results of these researches show that there is a great deal of variation in the behaviour of these inclusions in different forms. It is also known that the cytoplasmic inclusions behave in different ways even in quite closely related groups, so that yolk in one animal may have a totally different origin from that formed in the oocytes of another standing close to it in the evolutionary scale. Thus, in studying the course of oogenesis in any animal, it is often as useful to use totally unrelated forms for comparison as those which belong to the same class or phylum.

No previous work has been done on the cytoplasmic inclusions in the oogenesis of Oniscus, but it may be useful to summarise what is known to occur in certain other animals for the purposes of comparison.

One of the simplest cases of oogenesis is that which has been investigated by Gatenby and Nath in Lumbricus (3). In this case no yolk is present in the egg. The Golgi apparatus is at first juxtanuclear, and later passes out through the cytoplasm. The "yolk nucleus" of former workers is formed by a mass of mitochondria, which are demonstrable in all stages of the oocytes. No nucleolar activity is described. In this animal the cytoplasmic inclusions remain unchanged, except in position and numbers, throughout oogenesis, owing to the fact that none of them become transformed into yolk spheres.

The oogenesis of Lumbricus has also been studied in a recent paper by Harvey (13). This author has described the mitochondria in the later stages, and agrees with Gatenby and Nath in identifying them with the yolk nucleus of Calkins. His interpretation of the other cytoplasmic inclusions, however, differs widely from that of Gatenby and Nath. He states that yolk droplets are present in the oocytes from the very early stages, and that the Golgi bodies are never juxtanuclear, but are probably flat platelets arising from the cytoplasm. His conclusions have been fully discussed by Gatenby and Nath in their more recent paper, now in press. It will be sufficient to say here that their results, supported by the study of fresh mounts in weak osmic acid, and also of *intra-vitam* material, thus excluding all possibility artefacts, show that the bodies described by Harvey as yolk droplets are really the Golgi elements. These are juxtanuclear in the young stages, later fragmenting in the usual way. In Kopsch, Cajal, and Da Fano preparations, these bodies show the typical arrangement of dictyosome and sphere.

A somewhat more complicated oogenesis has been described in Patella by Ludford (10) and reinvestigated by Brambell in a recent paper (1). Ludford describes nucleolar extrusion during the process of yolk formation, but has not been able to prove that there is any direct connection between the events, although he suggests that this is the case. The yolk is formed directly under the influence of the Golgi bodies, but not from them; it is suggested that the yolk is formed by a storing up of fatty substances in fragments of archoplasm adhering to the Golgi rods. After the yolk is fully formed the Golgi rods break away from the spheres, and pass out to the periphery and in toward the nuclear membrane. The mitochondria take no active part in yolk formation. Brambell agrees with the above account, except as regards the behaviour of the mitochondria.. He describes these as swelling up during the growth stages, while also increasing rapidly in number by division. In Limnæa, in which a similar swelling of the mitochondria occurs, Gatenby and Woodger describe a corresponding shrinkage during the embryogeny, which evidently points to the using up of food-stuffs stored by the mitochondria during oogenesis. Thus, although the mitochondria cannot be regarded as yolk granules, since they retain their individuality, they certainly take part in the storing up of nourishment for the development of the embryo.

In Lithobius (8) the mitochondria take no part in yolk formation. The greater amount of yolk is formed from nucleolar extrusions, which bud and divide in the cytoplasm and then grow into yolk spheres. A certain amount of fatty yolk is also present; this has been claimed by Nath (12) to have been formed directly from the Golgi bodies, which swell up to form the yolk spheres.

In Ascidians, Hirschler (5) has described the Golgi bodies in a primary diffuse condition, later entering into a complex condition, after which they scatter to a secondary diffuse condition. In relation to these three phases of the Golgi bodies, he describes changes in the chromophility of the cytoplasm, from a primary oxyphilia to a basophilia, when the Golgi bodies are in the complex condition, and back again to oxyphilia when these bodies scatter once more. After this the Golgi bodies play a subsidiary part in yolk formation.

The mitochondria and yolk nuclei have a common origin from round bodies lying near the nucleus. The yolk nuclei are transitory cytoplasmic structures, disappearing completely during the growth of the oocyte. The mitochondria play the most important part in yolk formation. In the younger oocytes the mitochondria are concentrated in a ring round the nucleus; later they scatter through the cytoplasm, and grow directly into small yolk spheres. After the close of yolk formation the mitochondria tend to collect at the periphery of the cell. Yolk is formed at first directly from the mitochondria; then the Golgi bodies scatter through the cytoplasm and apply themselves to the yolk spheres, which then change their reactions, storing up fatty substances so that they become blackened in osmic acid. This process begins near the nucleus, the yolk at the periphery retaining its purely mitochondrial nature for a considerable time, and thus giving an appearance of zoning in the yolk, which recalls some of the stages in the oogenesis of Oniscus described below. No nucleolar budding has been observed in the oogenesis of Ascidians.

Material and Technique.

The ovaries were dissected out in salt solution, then transferred directly to the fixatives. For mitochondria and general cytoplasmic structure ovaries were fixed overnight in Champy's fluid, washed for several hours, and embedded. Sections were cut at 5 μ, and stained in iron hæmatoxylin.

Material was also fixed overnight in Bouin's fluid, and afterwards stained in iron hæmatoxylin, and in eosin and Ehrlich's hæmatoxylin for chromophility. Some difficulty was experienced in getting successful Golgi body preparations; Da Fano technique was quite unsuccessful, and only after many attempts by the Mann-Kopsch and Nassonov methods were good preparations obtained.

General Structure of the Ovary.

The ovaries are two in number, each having the form of a tube, and lying to the right and left of the gut, throughout the greater part of the length of the body. Each opens by a short tube toward the anterior end. The wall of the ovary is composed of a single layer of epithelium (Plate 2, fig. 8; *e*) outside of which is a thick membrane, smooth on the inner side, but covered by little projections on the outer surface (fig. 8, *w*).

The epithelium consists of columnar cells, the young oogonia; in places these appear to degenerate and slip away from the wall into the lumen of the ovary, their nuclei becoming shrivelled and staining darkly. Thus in some places the wall of the ovary appears to be formed only of the thick membrane mentioned above. The young oocytes slip into the lumen of the ovary, and almost immediately enter on the prophases of the first maturation division. No true follicle cells are present; the cells which degenerate after leaving the epithelium probably contribute to the nourishment of the oocytes, and are sometimes found close to them, but never in the definite investing sheath formed by true follicle cells. All sizes of oocytes are found free in the lumen of the ovary; but in the later stages, when they are of considerable size, they tend to fill up the whole lumen, so that the germinal epithelium is closely applied to the vitelline membrane, giving the appearance of a follicle in cross-section (fig. 8).

Behaviour of the Cytoplasmic Inclusions during Oogenesis.

(*a*) *Mitochondria.*—In the young oocytes, immediately after the first maturation division, the mitochondria are seen as a number of irregular granules scattered through the cytoplasm (Plate 1, fig. 1). They are larger at this stage than in any later phases, and as they grow smaller and more numerous progressively throughout the earlier stages of oogenesis, their growth does not keep pace with their multiplication by division, which is fairly rapid. In oocytes of the size shown in fig. 2 the mitochondria are still scattered, but in many oocytes of this age a part of the mitochondrial contents of the cell gathers in a ring round the nucleus, as shown (in a much later stage) in fig. 6. After this stage a definite zoning shows in the cytoplasm, the outer parts of which become pale and lose most of the mitochondria, which retreat with the darker-staining portion of the cytoplasm towards the nucleus. Fig. 3 shows an early stage in this process; in fig. 4 the zoning is more pronounced; and, finally, in fig. 5 the darker cytoplasm with its mitochondrial and yolk content forms a definite ring around the nucleus. During this process the mitochondria are becoming converted into yolk spheres, and owing to the great number of these in the cell, the true mitochondria, if any, remaining after the stage shown in fig. 5 become indistinguishable.

(*b*) *Golgi Bodies.*—In the young oocytes, the Golgi bodies are represented by a series of irregular granules found around the nucleus (fig. 7), which blacken with difficulty when treated with osmic acid. As growth proceeds these multiply and pass out through the cell, becoming, eventually, evenly distributed through the cytoplasm, except at the extreme periphery, where they are rather less frequent (Plate 2, fig. 9). They play an important part in the storing up of nourishment for the developing embryo, as they give rise directly to the fatty yolk.

(*c*) *Yolk Formation.*—The yolk in Oniscus is of two very distinct kinds, derived from different sources. In the young oocytes, when the mitochondria are still scattered, or just collecting in a perinuclear zone, certain of them can be seen to be swelling up, and at the same time acquiring a greater affinity for stains. These darkly staining spheres are the first appearance of the mitochondrial yolk (Plate 1, figs. 3, 4). As the mitochondria collect in a perinuclear zone they are accompanied by the yolk spheres. The peripheral portions of the cytoplasm from which the mitochondria have retreated are at first very pale (fig. 4), but as the perinuclear mitochondrial zone becomes smaller the outer cytoplasm regains its staining power and becomes more closely granular, while the ring round the nucleus becomes crowded with mitochondria and yolk spheres. At this stage a few of the mitochondria are found also outside this ring. During this process the fatty yolk has begun to form throughout the cytoplasm. As the oocyte enlarges the ring of mitochondrial yolk spheres surrounding the nucleus begins to retreat toward the periphery of the cell (fig. 6). The mitochondria are now once more evenly distributed throughout the cell, and continue to form yolk spheres, both in the outer zone and in the perinuclear region, but as these spheres increase in size they pass outward, so that most of the mitochondrial yolk spheres lying near the nucleus are quite small (Plate 2, fig. 8). Thus in the mature oocytes the mitochondrial yolk is concentrated in a peripheral zone.

The fatty yolk begins to form near the nucleus at quite an early stage. Its origin is beautifully seen in Nassonov preparations (fig. 9). Here we find Golgi elements scattered thickly in the cytoplasm. The Golgi bodies are of different sizes; the smallest are irregular black grains which are intensely and uniformly blackened by the osmic acid. These are the undifferentiated Golgi elements. From this undifferentiated condition all stages in the swelling up of the Golgi elements to form yolk spheres can be seen. As the Golgi bodies swell up, becoming loaded with nutritive matter, they acquire a clear central region in optical section, the peripheral parts alone becoming deeply blackened by the osmic acid. As seen in fig. 9 the Golgi bodies retain their distinctive characters and irregular shape until they have attained a considerable size, when they eventually round up and become yolk spheres. Owing to this fact no very small spheres of fatty yolk occur.

The Golgi yolk does not show well in Champy material except in the later stages. In some sections this type of yolk can be seen as vacuoles, or irregular lightly stained areas, but more often they seem to run together and form a few large irregular bodies (Plate 1, figs. 4, 5, 6, *g.y.*). In Bouin material it behaves in a similar way, but is not satisfactorily fixed even in the later stages.

In centrifuged oocytes, the mitochondrial yolk appears to be the heaviest cytoplasmic inclusion, and goes down to one side of the cell. The smaller spheres do not seem to be as dense as the larger ones, for they tend to remain distributed through the cytoplasm. This is what one would expect on the assumption that the proteid form of yolk is formed directly from the mitochondria; these originally light bodies would become progressively heavier as dense nutritive substances become stored up in them.

The Golgi yolk does not appear to be of the light fatty nature found in Patella, Saccocirrus and Lithobius, for in centrifuged oocytes it does not form a cap at the top of the cell, but remains distributed more or less evenly through the cytoplasm. In the centrifuged oocyte three zones can be distinguished (fig. 11), an upper zone containing only fatty yolk and Golgi bodies; a middle zone containing fatty yolk, Golgi bodies, mitochondria, and the nucleus; a lower zone containing a little fatty yolk and the bulk of the mitochondrial yolk. The smaller mitochondrial yolk spheres are, as already mentioned, found also in the middle and occasionally in the upper layer.

(*d*) *Nucleolus.*—In the oogonia of the germinal epithelium, the nucleolus, when distinguishable, is irregular in shape, and presumably engaged in budding; there is, however, no evidence that the budded-off particles are extruded into the cytoplasm. In the oocyte no evidence of nucleolar budding or extrusion has been found; the nucleolus shows no change, except an increase in size, during the whole course of oogenesis.

(*e*) *Chromophility of the Cytoplasm.*—In order to discover whether any changes in the chromophility of the cytoplasm occurred during oogenesis, material fixed in Bouin's fluid was stained in hæmatoxylin and eosin. Prepared by this method, the cells of the germinal epithelium are basophil; when they leave the epithelium and enter on the prophases of the first maturation division they gradually become amphophil, and shortly after the commencement of the growth period the oocytes become oxyphil, and remain so for the rest of their history.

Discussion.

A study of the many cases of oogenesis which have already been investigated shows that yolk formation is usually brought about chiefly by one or more of four factors—the mitochondria, Golgi apparatus, nucleolus, and ground cytoplasm. Cases have been described in which the yolk is derived directly from each of the first three, and in others (as, for example, Grantia), the reserve material appears to be formed under the direct influence of the ground cytoplasm. In the cases in which more than one factor is concerned in yolk formation, as in Ascidians, the process is not quite so easy to follow. Harvey, from his study of the oogenesis of Lumbricus, comes to the conclusion that yolk is not formed either from the Golgi bodies or mitochondria, and is therefore inclined to doubt the accounts of other authors who have studied groups in which such vitellogenesis occurs. A glance at the various types of oogenesis summarised at the beginning of this paper will show that, in work of this kind, it is impossible to make generalisations from a study of one form alone, since the greatest variation exists even in the oogenesis of allied animals. The same objection may be raised with regard to the views of Mayer, Rathery, and Schaeffer, who, from a study of hepatic cells (11), come to the conclusion that mitochondria are never directly transformed into other inclusions, and regard them as reversible products whose function is to keep the equilibrium between other products in the cytoplasm. A recent paper by Miss Guthrie (4) bears out this view of the nature of the mitochondria. Although this view may be correct in the cases cited, and the mitochondria may even have a universal function of maintaining the equilibrium between other cytoplasmic constituents, it is certain that in some cases mitochondria may become directly transformed into yolk spheres. This process has been clearly described by Hirschler in Ascidians, and it can also be followed easily in the material for this paper. As some of the mitochondria remain unaltered, if we assume the truth of the equilibrium theory we may infer that these act in the way it suggests, while the surplus mitochondria take part in yolk formation.

The definite zoning of the mitochondrial yolk in the oocytes of Oniscus is an interesting and rather unusual phenomenon. The stages shown in figs. 5 and 6 strongly recall the appearance shown in Hirschler's Ascidians (5, figs. 23 and 25), in which a zone of blackened yolk is seen round the nucleus, while the yolk spheres at the periphery stain bright red. The cases are, however, not comparable. In Oniscus the zoning is due to the definite collecting of the mitochondrial yolk in a layer at the periphery of the oocyte, while the Golgi yolk is distributed throughout the cytoplasm, but more plentiful near the nucleus. In Ascidians all the yolk spheres are at first mitochondrial, and stain red with fuchsin; then the Golgi elements apply themselves to their surfaces, and influence their development in such a way that they become loaded with fatty material which blackens with osmic acid. The Golgi bodies spread slowly outward through the cytoplasm, so that the area of yolk coming under their influence increases gradually, and thus the zoned appearance arises in quite a different way to that found in Oniscus.

The formation of fatty yolk from the Golgi elements in Oniscus is strictly comparable to the process described by Nath in Lithobius (12), and probably also to the case of Saccocirrus (2), since the Golgi bodies swell up directly to form the yolk spheres. The condition in Patella is rather more complex, the Golgi bodies retaining their individuality, and, after they have been instrumental in forming the yolk spheres, breaking away from them completely. The absence, in the oogenesis of Oniscus, of the nucleolar activity characteristic of many Arthropods, is rather unexpected. Nucleolar yolk formation has been described in various Hymenoptera and in Periplaneta by Hogben (6 and 7), and occurs also in Lithobius (8); Gatenby has also described the same process in Saccocirrus (2). In Peripatus (9) I find nucleolar budding, and, almost certainly, extrusion, during oogenesis, but have not been able to connect it directly with yolk formation. The inactivity of the nucleolus in Oniscus oocytes is but another illustration of the fact that even closely related forms often show striking differences in their cytology.

Summary.

1. Yolk is of two kinds, one directly derived from the mitochondria, the other from the Golgi bodies. The mitochondrial yolk is at first rather diffuse, then retreats to a zone around the nucleus, and then passes outwards to a peripheral zone. The Golgi yolk is evenly distributed through the cytoplasm.
2. The mitochondria are at first scattered, then tend to collect round the nucleus. Later they become scattered again.
3. The Golgi bodies are concentrated near the nucleus in the young oocyte, and later pass out through the cytoplasm, where many of them swell up to form yolk spheres.

4. The oogonia and youngest oocytes are basophil, but the latter become oxyphil early in their development, and remain so until the close of oogenesis.

5. No nucleolar activity has been described in the oocytes of Oniscus.

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DESCRIPTION OF PLATES 1 AND 2.

LETTERING.

e, germinal epithelium of ovary wall; *g*, Golgi bodies; *g.y.*, Golgi yolk; *m.*, mitochondria; *m.y.*, mitochondrial yolk; *n.*, nucleus; *w.*, outer membrane of ovary wall.

PLATE 1.

FIG. 1. Young oocyte showing scattered mitochondria.

FIG. 2. Older oocyte, mitochondria still scattered.

FIG. 3. Older oocyte; mitochondria collecting to perinuclear zone; beginning of mitochondrial yolk formation.

FIG. 4. Later stage; zoning marked in cytoplasm; mitochondrial and Golgi yolk present.

FIG. 5. Later stage showing mitochondrial yolk concentrated in perinuclear zone.

FIG. 6. Older oocyte, mitochondrial yolk concentrated in peripheral zone.

FIG. 7. Golgi bodies perinuclear in young oocyte.

PLATE 2.

FIG. 8. Nearly mature oocyte; Golgi yolk in perinuclear zone; mitochondrial yolk peripheral.
FIG. 9. Part of cytoplasm of oocyte, showing Golgi bodies swelling up to form yolk.
FIG. 10. Oogonia from germinal epithelium showing Golgi bodies.
FIG. 11. Semi-diagrammatic centrifuged oocyte.
FIG. 1–6 and 8 from material fixed in Champy's fluid, stained iron hæmatoxylin; Figs. 7, 8, and 9–11 from Nassonov material.

Magnification: Figs. 1, 2, 7, 9 and 10 × 2300; Figs. 3, 4, 5, 6 and 8 × 1200.

The Relation between Oxygen Requirement and Speed in Running.

By R. M. SARGENT.*

(Communicated by Prof. A. V. Hill, F.R.S.—Received February 19, 1926.)

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A. V. Hill and his co-workers (1) have emphasised that the relation existing between the average speed at which a race can be run and the distance to be covered depends mainly upon three factors: (*a*) the oxygen requirement of the exercise, (*b*) the oxygen intake of the subject while running, and (*c*) the oxygen debt incurred by the subject. Their experiments have shown that the general type of relation that exists between speed and oxygen requirement is likely closely to resemble that found by Furusawa (2) in the case of standing-running. The results obtained hitherto, however, are of limited application. In the first place, their observations were made on a variety of subjects who used a track which was too small to permit of a high speed being readily obtained, and, in the second, their subjects were compelled to carry a large Douglas bag during the performance of the exercise. The conical Douglas bags used to collect the expired air during exercise weigh, complete with mouthpiece and rubber connections, about 10 lb.; in addition to their weight they are extremely cumbersome and hinder the free action of the subject to a marked degree.

In order to arrive at some more definite conclusions as to the nature of this relationship, the whole question was re-examined, with the idea of obtaining accurate data, not hitherto available, on *one* trained subject under carefully controlled conditions.

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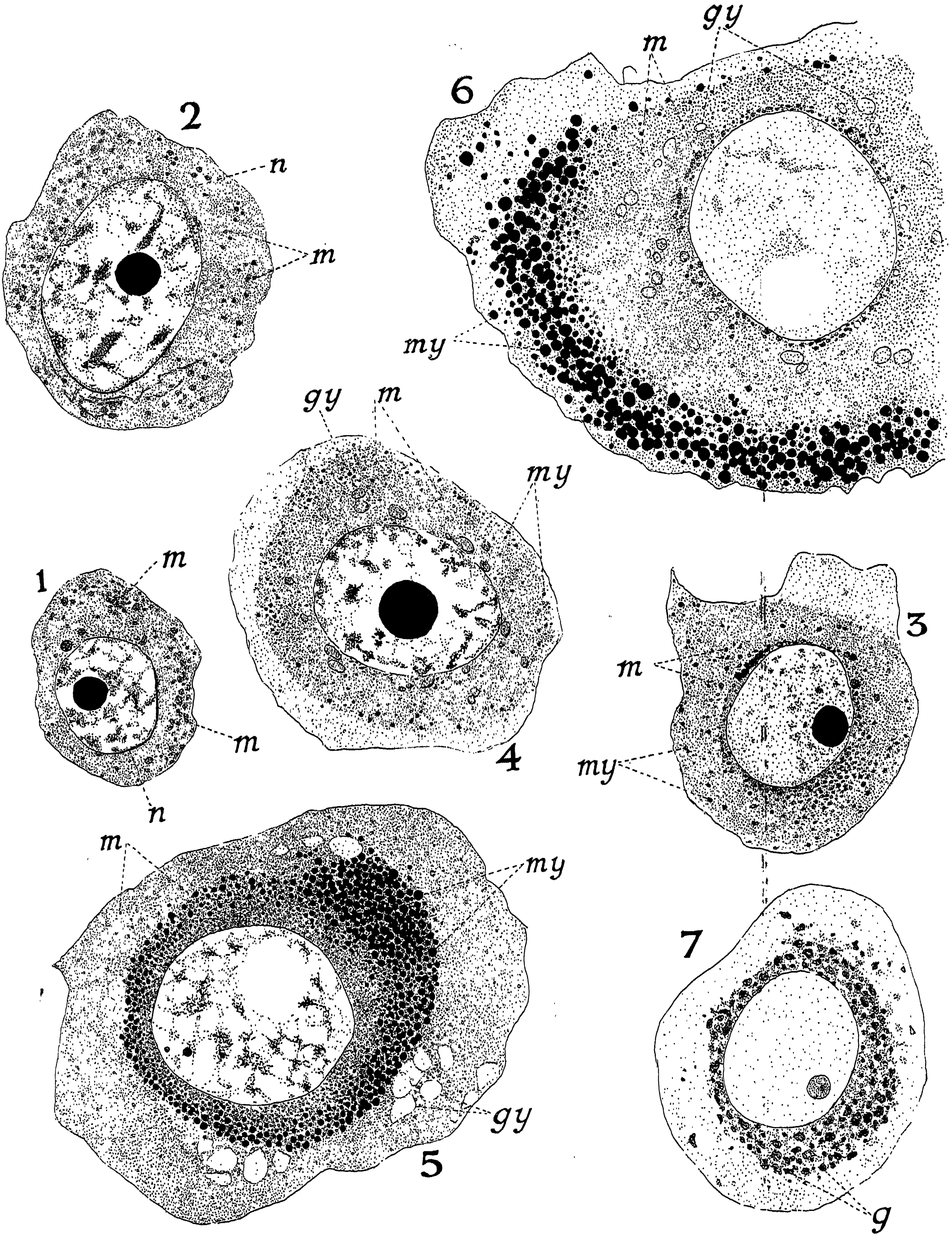
* Working for the Industrial Fatigue Board of the Medical Research Council.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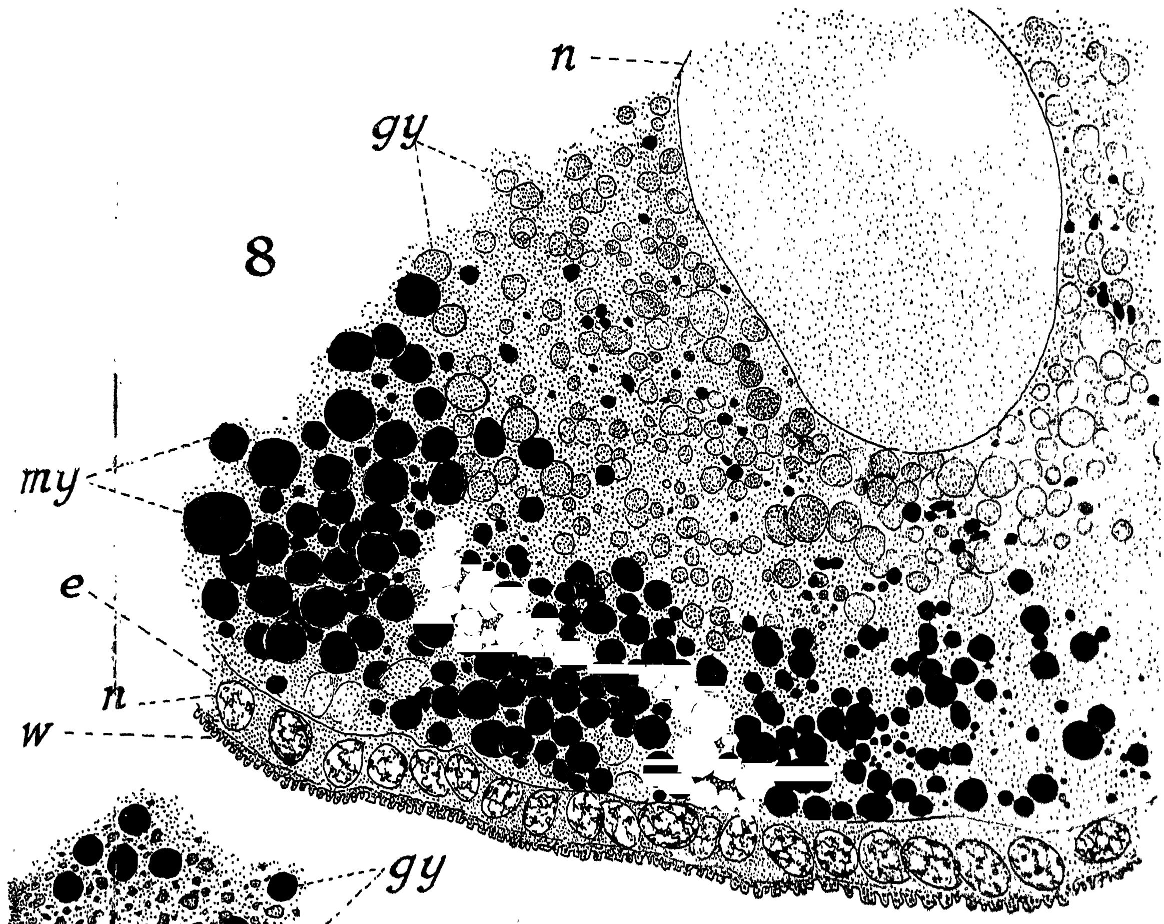

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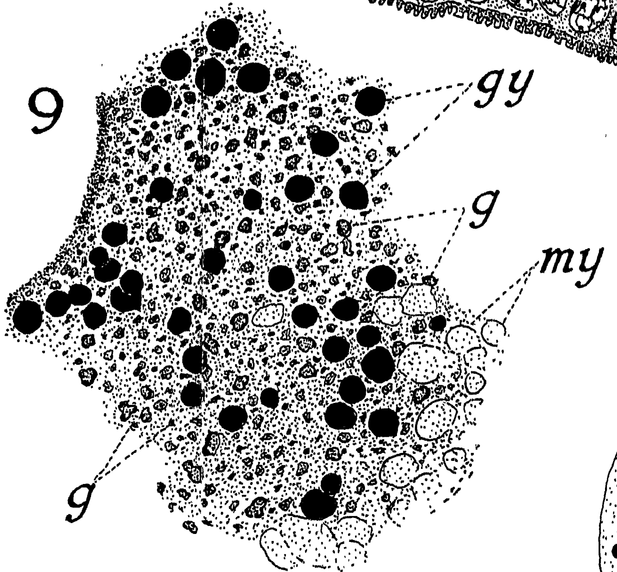

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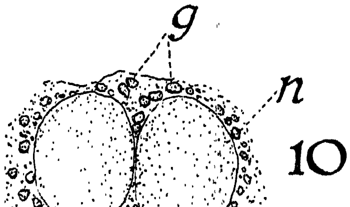

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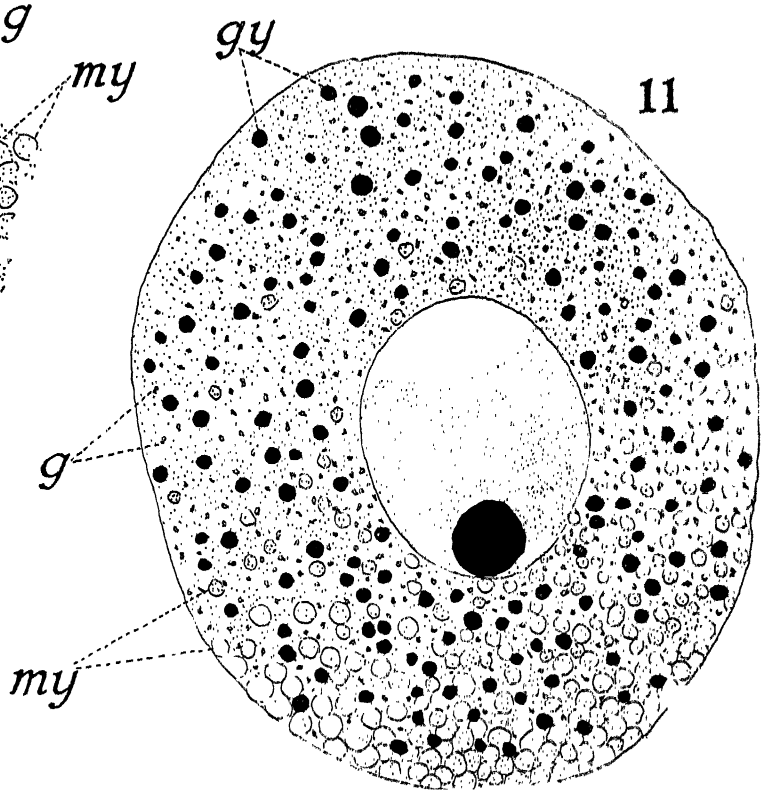

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The observations here detailed were so arranged that the nearest approach was obtained to the actual conditions of track work, consistent with experimental requirements. To obviate errors due to carrying a bag, the subject held his breath while running, placing the mouthpiece in his mouth and breathing into a bag immediately on the conclusion of his run.

The subject, N., is an experienced runner, and is a capable performer in both sprint and long-distance work; his best times for the various distances, given in Table VII, approximate to the standard of Inter-University and of Championship performances. Sir Thomas Lewis very kindly examined him for me, and reported that his heart action is perfectly normal, his heart being, if anything, somewhat small for a man of his size.

Procedure.

The purpose of most of the experiments was to determine the total oxygen requirement of running a given distance (120 yards) at a series of different speeds. The technique employed was that of the Douglas bag, as used by Hill and others in the experiments referred to above.

Determination of the Total Oxygen Requirement.—Most of the observations were made in the evening, some hours after the subject had had a very light tea. At the beginning of each series of experiments a collection of the subject's expired air was made for ten minutes after he had been thoroughly rested for at least half an hour in a recumbent position; this served as a "basal" for that particular evening.

After the "basal" collection had been sampled and measured, the subject walked slowly to the starting-point, and after a rest there of some minutes, took up his starting position, using foot-holes that had previously been dug for him. Two seconds after the order "get set" had been given, and just before the pistol was fired, he inspired as deeply as possible. This breath he held over the whole distance (120 yards), which he ran at a pre-arranged speed; this speed he endeavoured to keep as constant as possible. The time was taken in the usual manner by an observer at the finishing-point.

After passing the finishing point, he had a "pull-up" of 20 yards, in which he fixed the nose-clip and ran to his resting place; there he immediately placed the mouthpiece in his mouth, and started expiring into the bag, after which he lay down and was covered with blankets. The "pull-up" was made to involve as little effort as possible, so as not to add too much to the energy cost of the run under consideration.

The expired air was passed through a meter on its way to the bag, and collection was continued till the minute ventilation showed a near approach to the "basal" level. The contents of the bag were then sampled and measured.

By the method outlined above, all errors due to the effort of carrying a bag are eliminated, and the energy utilisation measured is only that involved in the actual performance of the exercise, apart from the small amount involved in the "pull-up." The holding of the breath over so short a distance usually requires little effort, and caused the subject no discomfort. As a matter of fact, some trainers recommend their pupils to do so over the shorter distances.

Two samples were taken from each bag, and each analysed twice on automatically operated Haldane apparatus; pairs of samples in which the results of the two analyses differed by more than 0·05 per cent. were rejected. In Table I, the results of all but three of the experiments performed are included; in these three experiments the results were disregarded, as loss of expired air was known to have occurred, either in the actual running or else in the fitting on of the mouthpiece.

The results—oxygen requirement as a function of speed—are plotted in fig. 1. Curve 1 refers to the oxygen required to complete the given task, *i.e.* the oxygen requirement *per* 120 *yards run*: curve 2 to the oxygen requirement *per minute*. Further discussion is reserved till later. No account is taken in Table I. and fig. 1 of the energy involved in starting and stopping.

Table I.—Subject N. Wt., 63 kilos. Vital capacity, 4·4 litres. Average resting oxygen intake, 0·255 litre per min. Distance run, 120 yards, with a "pull-up" of 20 yards.

Speed.	Oxygen requirement per 120 yards.	Oxygen requirement per minute.
Yards per second.	Litres.	Litres.
5·56	1·83	5·08
6·45	2·71	8·75
7·15	2·94	10·50
7·24	3·08	11·13
7·70	3·85	14·81
8·40	4·33	18·17
9·10	6·27	28·46
9·23	7·36	33·96

Determination of the Maximum Oxygen Debt.—The maximum oxygen debt that this subject could incur was measured in the usual manner. He ran a certain distance "all-out" and immediately on finishing the run put on the mouthpiece and noseclip, and expired into a bag, as previously described.

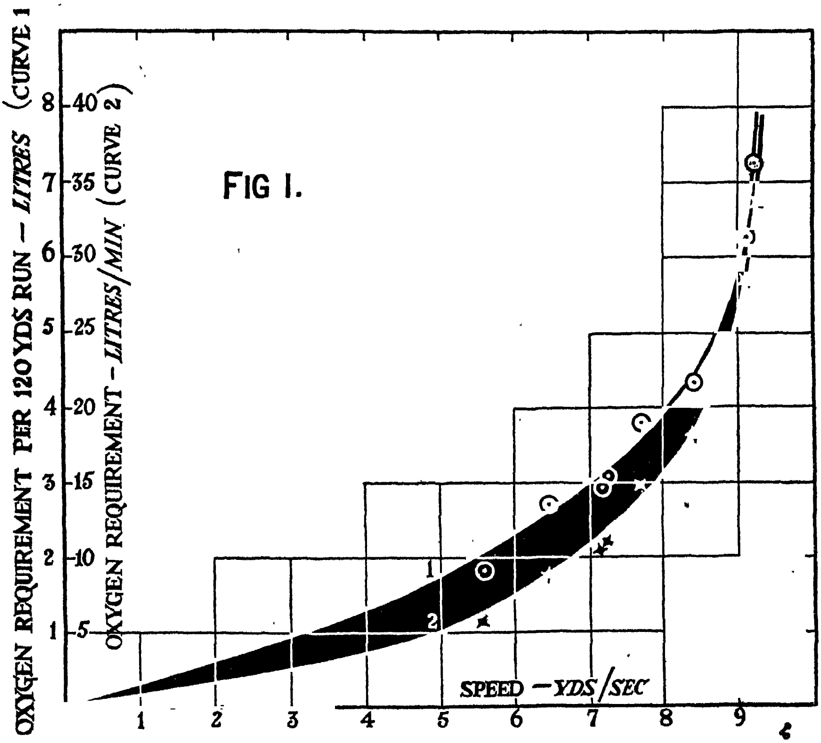

Fig. 1.—Oxygen requirement of running as a function of speed. Results as observed, uncorrected for "start" and "pull up." Curve 1 and left-hand vertical scale, oxygen requirement per 120 yards run. Curve 2 and right-hand vertical scale, oxygen requirement per minute.

Owing to the extremely unsettled state of the weather during the summer of 1925, and to other difficulties, it was impossible to make more than three determinations of the maximum oxygen debt; these are given below.

Table II.

Experiment A.—Running 300 yards in 35·1 secs.; track wet and heavy. "Pull-up" of 30 yards, time 3·8 secs. Resting $CO_2/O_2 = 143/208 = 0 \cdot 7$. Recovery 41 mins.; oxygen debt 16·3 litres = 0·26 litre per kilo. of body weight.

Experiment B.—Running fast round quadrangle for 2·5 mins., followed by "standing-running" while the mouthpiece was being put on. The subject showed signs of extreme exhaustion. Resting $CO_2/O_2 = 189/229 = 0 \cdot 83$. Recovery 50 mins.; oxygen debt 13·7 litres = 0·217 litre per kilo. of body weight.

Experiment C.—Running 440 yards in 55 secs.; track wet and heavy. "Pull-up" of 30 yards. Resting $CO_2/O_2 = 216/281 = 0 \cdot 77$. Recovery 60 mins.; oxygen debt 10·45 litres = 0·165 litre per kilo. of body weight.

The greatest oxygen debt was obtained after a three-hundred yards run and amounted to 16·3 litres. This value, reckoned per kilogram of body weight, is much greater than that found by A. V. Hill and other observers for any of their subjects. Although the experiment giving this value appears to be perfectly straightforward, the value itself is so unprecedented, and is associated with such an abnormally low " basal " R.Q. (suggesting an error in the " basal " measurements) that it has been neglected in the calculations in the final part of this paper. The maximum oxygen debt is taken as 13·7 litres.

Determination of the Maximum Oxygen Intake.—The subject ran various distances at different speeds, which he endeavoured to keep as constant as possible; throughout the run he wore the mouthpiece, to which was attached a length of flexible rubber tubing, and carried the noseclip in his hand. Twenty yards before the finish of the distance proper he was joined by the experimenter, who affixed to the tube a small 100-litre bag, carrying a three-way tap communicating initially to the air, and ran, carrying the bag, by the subject's side for the rest of the distance; at the same time the subject closed his nose with the noseclip. Directly the finishing-point was reached, the observer turned the tap, putting the interior of the bag in communication with the mouthpiece, the observer and subject continuing to run. The timer accompanied them over the collection-distance, receiving a signal at the beginning and at the end of collection, which was made in each case over a distance of 220 yards (*e.g.* if the subject was to run three-quarters of a mile, he would run three quarter-mile laps, and collection would be made over an additional half-lap after the completion of the third lap).

The greatest value obtained for the oxygen intake was that after a run of three-quarters of a mile, and amounted to 3·75 litres per minute.

Table III.—Maximum Oxygen Intake: Two Typical Experiments.

Experiment D.—" Standing-running " at 240 steps per min. for 257 secs. " Steady state " level reached 120 secs. after the commencement of the exercise, at an *oxygen intake* level *of* 3·04 *litres per min.*

Experiment E.—Running three quarter-mile laps at an approximately constant speed of 5·25 yards per sec.; final rate of *oxygen intake* 3·75 *litres per min.*

This subject finds " standing-running " extremely difficult, and is unable to keep the exercise standardised for any length of time. During actual running, the feet are just swung clear of the ground, and any energy expenditure from raising them too high is avoided. Possibly some established inhibition operates while the subject is endeavouring to do " standing-running."

In the calculations to be made below it is necessary to know not only the maximum oxygen intake, once a "steady state," or apparently "steady state," has been attained, but also the maximum amount of oxygen that can be taken in during various intervals of time (*e.g.* of half or one minute) starting from rest. For this purpose it is necessary to determine the rate of rise of the oxygen intake from the commencement of exercise. Experiment D, Table IV, records observations made by the method described by Furusawa (3). The oxygen intake was here calculated as the average per minute over varying successive intervals, and was plotted in a figure to the midpoint of the intervals. As we are concerned in our later calculations only with the "excess" oxygen intake, it is necessary to subtract the "basal" oxygen intake from our actual observations.

Table IV.

Experiment D.—"Standing-running" at 240 steps per minute for 257 secs. "Basal" oxygen intake, 0·28 litre per minute.

Time: seconds	25	55	84	120	150	212
Rate of excess O_2 intake: litres per minute	1·48	1·93	2·58	2·76	—	2·61

From the above, for the purpose of the discussion given below, the following have been calculated for an assumed final maximum rate of oxygen intake of 3·75 litres per minute (*see* Experiment E, Table III), less the "basal" value, which is taken on the average to be 0·26 litre per minute, *i.e.* to a final maximum level of 3·49 litres per minute.

Time: minutes	0·5	1·0	2·0	3·0
Rate of excess O_2 intake, litres per minute; smoothed values from curve	1·52	2·24	2·74	2·80
Rate of excess O_2 intake, calculated to maximum intake of 3·49 litres per minute	1·89	2·80	3·43	3·49

Discussion.

It will be seen from curve 1 of fig. 1 (oxygen requirement against speed in yards per second) that at the higher speeds the oxygen requirement of the exercise shows a relatively much greater augmentation for a small increase in speed than at the lower speeds; at low speeds the oxygen requirement is comparatively small, whilst at the higher speeds it exhibits a very rapid and disproportionate rise. The addition by N. of even one foot per second to his highest speed of 9·23 yards per second (under the conditions of training, etc., obtaining in these experiments) would appear to be almost impossible, as in this region the curve becomes practically asymptotic vertically.

The highest speed at which N. can run without incurring any increasing oxygen debt (*i.e.* at which his oxygen requirement can be entirely satisfied by the "steady state" level of his oxygen intake) is that which involves an "excess" oxygen requirement of 3·49 litres per minute: this corresponds to a speed of 5·14 yards per second (from curve of corrected oxygen requirement, *see* fig. 2 below). At this speed (apart from other factors) N. would run one mile in 5 mins. 47 secs. and ten miles in about 58 mins., so that it appears impossible for him to run any distance in a good time without incurring an oxygen debt.

Before proceeding farther, it is necessary to take account of a factor hitherto neglected, viz., the amount of energy utilised in the initial acceleration and the final retardation—the "start" and the "pull-up." This energy, which is only a small—but not negligible—fraction of the whole, can be allowed for approximately on the following assumptions :—(*a*) that the total energy used in starting, or in stopping, is proportional to the kinetic energy of the runner, as measured by $\frac{1}{2}MV^2$, where M is his mass and V his average velocity throughout the run ; and (*b*) that this kinetic energy is produced, or destroyed, with an "efficiency" of 20 per cent., as found in most muscular movements. Thus starting will, on these assumptions, involve a utilisation of total energy of $5 \times \frac{1}{2}MV^2$ ergs (where M is mass in grammes and V velocity in centimetres per second), as also will stopping, the total in "start" and "pull-up" together being $5MV^2$ ergs. It is realised that this allowance is only approximate, but since it is a comparatively small correction, no very serious error will be involved. This energy can then be interpreted in terms of oxygen usage, assuming 1 litre of oxygen to be the equivalent of $2 \cdot 11 \times 10^{11}$ ergs (*see* below), and we may calculate the simple formula for N. (63 kilos. weight) :—

Total oxygen requirement (litres) of starting and stopping $= 0 \cdot 0125\ V^2$, where V is now the average speed attained in yards per second. The following corrected table then results :—

Table V.

Speed.	O_2 requirement observed.	Correction.	Net O_2 requirement.	Net O_2 requirement per min.	Rate of energy expenditure.
Yds. per sec.	Litres.	Litres.	Litres.	Litres.	Horse-power.
5·56	1·83	0·39	1·44	4·0	1·88
6·45	2·71	0·52	2·19	7·1	3·34
7·15	2·94	0·64	2·30	8·2	3·86
7·24	3·08	0·65	2·43	8·8	4·14
7·70	3·85	0·74	3·11	12·0	5·65
8·40	4·33	0·88	3·45	14·5	6·82
9·10	6·27	1·03	5·24	23·8	11·21
9·23	7·36	1·07	6·29	29·0	13·70

The last column is calculated as follows : 1 litre of oxygen per minute used in the combustion of carbohydrate will supply energy at a rate equal to 0·471 horse-power ; exercise requiring, per minute, the consumption of x litres of oxygen is calculated as requiring $x \times 0 \cdot 471$ horse-power of total energy expenditure ; this represents the actual horse-power of the breakdowns concerned only on the assumption that the oxygen is used during the exercise (*see* text, p. 18) ; actually only two-fifths of this energy is used during the actual effort, the rest appearing in recovery.

The "net" figures given in this table and in fig. 2 refer to the oxygen required for *running only* at the speeds indicated, as distinguished from the oxygen required for start, run, and "pull-up" taken together, and shown in Table 1 and in fig. 1.

Curve 1 of fig. 2 (corrected oxygen requirement per 120 yards against speed in yards per second) gives the approximate relation that the oxygen requirement for running a given distance increases as the 2·8th power of the speed; curve 2 shows that the oxygen requirement *per minute* increases as the 3·8th power of the speed. The reasons for this type of relationship between oxygen requirement and speed are complex. If the oxygen requirement per step, or per yard, were constant, then a direct proportion would exist between oxygen requirement per minute and speed. The probable reason, in general, for the relation that exists is the diminution of external work done with increase of speed of movement, as described by A. V. Hill (4), Lupton (5), and Gasser and A. V. Hill (6). At high speeds, to secure the same external result, a greater effort must be made than is necessary at the lower speeds. At high speeds, moreover, the external result has actually to be greater, the momentum of the limbs being extremely large, and having alternately to be created and destroyed by active muscular effort.

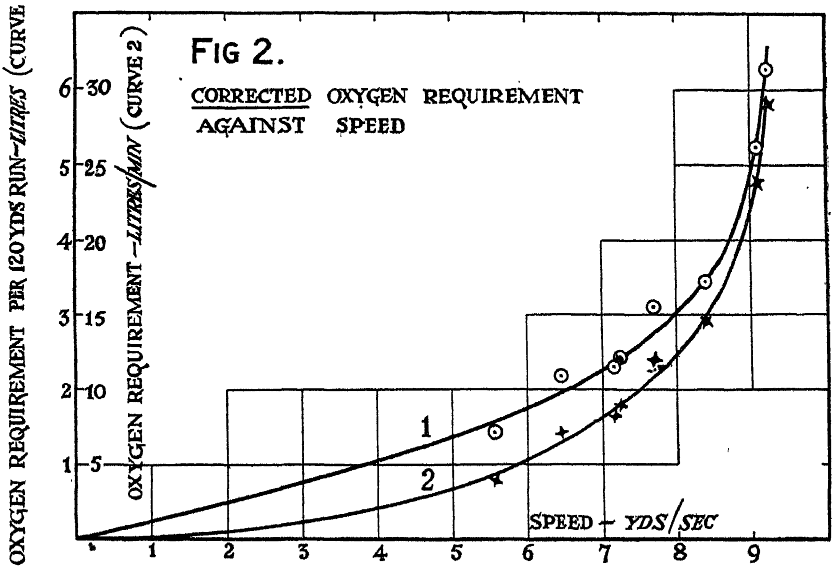

FIG. 2.—Oxygen requirement of running as a function of speed. Curve 1 and left-hand vertical scale, oxygen requirement per 120 yards run. Curve 2 and right-hand vertical scale, oxygen requirement per minute. The results shown have been corrected for the energy involved in "start" and "pull up" and represent the net requirement of running only.

For severe exercise of short duration, such as we are here considering, Furusawa (7) has shown that the respiratory quotient of the excess metabolism resulting from the exercise approximates to unity, *i.e.* that during, and after, the exercise the energy requirements of the body are entirely met by the oxidation of carbohydrate. Assuming then that during exercise and recovery each litre of oxygen used represents an energy expenditure of 5·05 Calories, derived from the combustion of carbohydrate, we may calculate the energy cost of running a given distance at any speed on our curve.

We have

$$\begin{aligned} 1 \text{ litre of oxygen} &\equiv 5\cdot 05 \text{ Calories.} \\ &\equiv 2\cdot 11 \times 10^4 \text{ joules.} \end{aligned}$$

Thus the maintenance of an oxygen consumption of 1 litre of oxygen per minute implies the expenditure of energy at a rate equivalent to $(2\cdot 11 \times 10^4)/(60 \times 10^3)$ or 0·352 kilowatt, which is equivalent to 0·471 horse-power. Thus, if it were possible for N. to run continuously at a speed of 9·23 yards per second, his corrected oxygen requirement at that speed being 29 litres per minute, he would require a total energy expenditure equivalent to 13·7 horse-power. In this sense, in running at 9·23 yards per second, he is incurring an oxygen debt at a rate equivalent to 13·7 horse-power (*see* Table V). During the exercise itself, which, of course, cannot be maintained for long, only two-fifths of this energy is liberated, since the ratio of recovery heat production to initial heat production is as 150 : 100 (Hartree and A. V. Hill (8)). The remaining three-fifths is liberated in recovery. Thus in running at top speed the rate of contemporary energy liberation is about 5·5 horse-power, and similarly at other speeds.

The matter can be expressed in another way : 1 litre of oxygen being the equivalent of 5·05 Calories, is the equivalent also of 2,140 kilogram-metres of work. The weight of N. being 63 kilos., this is equivalent to raising his body 34 metres into the air, so that an oxygen requirement of 7·36 litres for running 120 yards (including starting and stopping) at his maximum speed is the equivalent of 15,750 kilogram-metres of work, or of raising his body 250 metres. Actually, of course, if employed in doing mechanical work in the body, the mechanical efficiency with which the energy would be utilised would be only about 20 per cent. ; thus the *mechanical* energy liberated by N. when sprinting at top speed is equivalent to about 3·2 horse-power, and the mechanical work done in running 120 yards is the equivalent of raising his body about 50 metres above the ground.

The energy cost of this type of vigorous exercise of short duration is extremely high. The curves of fig. 1 and of fig. 2 emphasise the very uneconomical nature of such rapid and violent movement, and show why it can be maintained only for such a very short time.

With the data at our disposal, it should now be possible to arrive at some idea of the physiological limitations and capabilities of our subject. We have (*a*) a curve (fig. 2) of oxygen requirement per minute against speed, (*b*) information as to the subject's maximum oxygen intake per minute, and (*c*) some idea as to the maximum oxygen debt that he can incur. From these we can arrive at the figures of Table VI, which express the amount of energy of which the subject can possibly avail himself in various short intervals of time.

Table VI.

Time, T : mins.	½	1	1½	2	2½	3	4	5	10
Max. O_2 debt, D : litres	13·7	13·7	13·7	13·7	13·7	13·7	13·7	13·7	13·7
O_2 intake, I : litres	0·52	1·71	3·23	4·90	6·63	8·67	11·85	15·33	32·73
Total O_2 available per min. $(D + I)/T$: litres	28·44	15·41	11·29	9·30	8·13	7·46	6·39	5·81	4·64
Corresponding speed : yds. per sec.	9·22	8·46	7·76	7·30	6·97	6·77	6·44	6·23	5·75
Distance covered : yds.	276	508	698	876	1045	1218	1545	1869	3450

Of the items given in Table VI, one only—that headed " oxygen intake "—requires further explanation ; reference to this is made in the note given in Table IV, the total area of the oxygen intake curve up to any given moment being taken as giving the maximum oxygen available up to that moment.

If the subject after running for any given time has completely exhausted himself, we may assume that he has incurred as large an oxygen debt as he is able to do. Otherwise he could have run faster. While running, he derives his energy from two sources : (1) from his oxygen intake, and (2) from the breakdowns which lead to, and are measured by, his oxygen debt, which has to be met in the following period of recovery. Over a sprint distance he will depend mainly upon his capabilities of creating an oxygen debt, which will mount up rapidly, the amount of oxygen which he obtains in so short a time from his oxygen intake being negligible in comparison with it. Over longer distances he will depend more upon the oxygen that he inspires, and his oxygen debt will rise only slowly to its maximum, which we may assume to be reached at the conclusion of the run. By taking the sum of these two quantities (maximum oxygen debt and maximum excess oxygen intake over a definite time period) as representing the total amount of oxygen of which he can avail himself, we can calculate his energy expenditure in terms of oxygen requirement over the time period under consideration. The results so obtained are given in the table as "total oxygen available per minute"; the speed which necessitates such an oxygen requirement can be read off from the curve of fig. 2. By multiplying the speed so obtained by the time period under consideration, we can arrive at the distance which N. can cover in that time. The figures so determined are given in Table VI and in fig. 3. From the curve of fig. 3 we can determine, solely by considerations of the oxygen requirement, etc., of our subject, the times that he should take over various specified distances. These, together with N.'s best times (either actually observed, or estimated by him some months before, and quite independently of, our calculations) are given in Table VII. Considering the number of

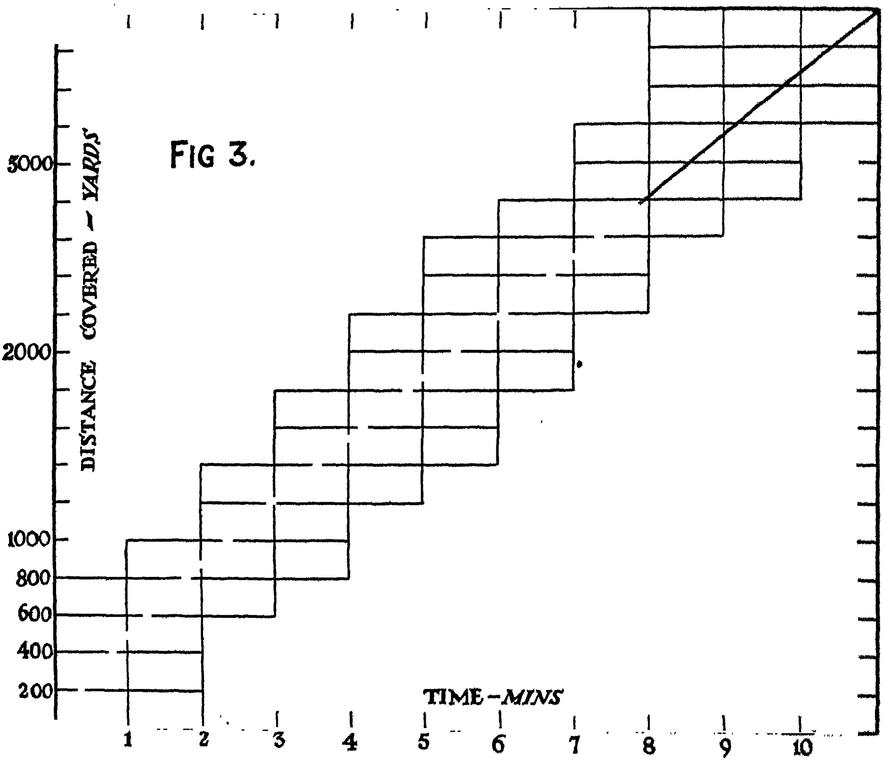

Fig. 3.—Maximum distance which can be covered by the subject N. in the time given by the abscissa. Results calculated from the data of Tables V and VI. For comparison with observations *see* Table VII.

assumptions and observations that it has been necessary to make, the agreement is striking; there would appear to be little doubt that in a general way *speed in running is determined mainly by considerations of energy utilisation,* such as those advanced above.

Table VII.

Distance	300 yds.	¼ m.	⅓ m.	½ m.	¾ m.	1 m.	2 m.
Deduced time	33·3″	51·5″	1′ 12·5″	2′ 1″	3′ 19″	4′ 41″	10′ 12″
N.'s best time, observed or estimated*	34″	51·4″	1′ 15″	2′ 2″	—	4′ 40″ *	10′ 10″ *

Distances less than 300 yards are not given, as our subject is unable to incur his maximum oxygen debt in a shorter distance.

The true oxygen requirement of running, say, 50 yards at a certain speed could be ascertained —at any rate, in theory—by determining the difference between the oxygen requirements of (*a*) running 100 yards and (*b*) running 50 yards at the same speed, both observations being uncorrected for "start" and "pull-up," the latter being in both cases over the same distance. The value so obtained would obviously represent the true oxygen requirement for running 50 yards at that particular speed. Owing to lack of time, this scheme was not tested; in any case, it seems almost impossible to hope that any subject, however experienced, would be able to "hit off" exactly the same speed in any two experiments; consequently, it would be necessary to make a complete curve like that of fig. 1 for each distance, and to interpolate the difference between them.

It may be noted that, for simplicity in calculation, the energy required in starting is not allowed for in the calculations of Table VI: over the distances involved the resulting error is negligible.

Summary.

1. A modification is described of the usual method of determining the energy expenditure during running at various speeds. This method makes it unnecessary for the subject to wear a mouthpiece, or to carry a Douglas bag, during the actual performance of the exercise, which can take place under natural conditions on a running track. The results obtained show the actual energy expenditure involved in the performance of the exercise, together with that involved in the "start" and in the "pull-up."

2. It is shown that for the subject of these experiments to run 120 yards in 13 seconds necessitates an energy expenditure equivalent to an oxygen requirement of 29 litres per minute, or to 13·7 horse-power. Attention is drawn to the extreme energy cost of rapid and vigorous exercise of short duration.

3. An approximate means of allowing for the energy utilised in the "start" and in the "pull-up" is adopted, and the net oxygen requirement of running only is given in a curve as a function of the speed.

4. The general relation between speed in running and oxygen requirement is as follows :—The oxygen requirement per 120 yards increases about as the 2·8th power of the speed, whilst the oxygen requirement per minute increases approximately as the 3·8th power of the speed.

5. The application of the physiological results to the calculation of the optimum performance of the subject for any distance is described. The calculated optimum performances agree well with those actually recorded (or estimated) by the subject, over the range 300 yards to 2 miles.

It is concluded that energy expenditure is the chief consideration in determining athletic performances.

In conclusion, I would like to express my sincere thanks to Prof. A. V. Hill, who suggested this piece of work to me, for his very kind help and advice during the performance of these experiments, to Mr. Furusawa and to Dr. Kubo for their assistance at Queen's Club, to the Secretary and to the Manager of Queen's Club for their kindness in allowing me to make use of their ground, and, finally, to my subject, Mr. S. E. Nelson, to whom the success of these experiments is mainly due.

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Rouget Cells and their Function.

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Of recent years, owing to the work of Krogh and his collaborators, the idea that the contractility of blood capillaries resides in certain adventitial cells has been brought into prominence and has gained a wide acceptance.

Vimtrup (5, 6, 7) succeeded in displaying, by histological methods, cells applied to the walls of capillaries. He stated that these cells were contractile, and that, by the shortening of their elongated processes, the capillary lumen was narrowed. These results were obtained, for the most part, in the frog, although analogous cells were described as existing on the capillaries of the mammalian skin.

It seemed desirable to verify these observations for mammalian capillaries before assuming that such cells were the agents in producing capillary contraction in that type of animal. For the purpose of experiment, the mesentery of the cat and dog was selected.

I. HISTOLOGICAL OBSERVATIONS.

Technique.

A method of staining has been elaborated by means of which a fairly specific staining of the vascular tree and the cells in its immediate neighbourhood could be obtained.

The intestines of a cat or dog, together with the complete mesentery, were rapidly removed from the chloroformed animal. A cannula was inserted into the mesenteric artery, and another into the largest branch of the portal vein. The intestines were kept warm by saline swabs in order to have them in a flaccid condition. The blood was rapidly washed out by the perfusion of 0·9 per cent. saline solution. Next, a 4 per cent. solution of formaldehyde was perfused and allowed to remain in the vessels for five minutes. This was then washed out with water.

Staining was also carried out by the vascular route. Gentian violet, basic fuchsin and Heidenhain's iron hæmatoxylin gave good results when used in this way. In the case of the latter stain, the mordant portion was first perfused, and, after filling all the vessels, was left in for 10 minutes. This was then washed out with water, and the hæmatoxylin perfused and also left in for 10 minutes. Pieces of mesentery, stained by this or by other methods, were excised and differentiated in watch-glasses, the differentiation being controlled microscopically.

Dehydration, clearing (usually in clove oil), and mounting were conducted on standard lines. By these methods preparations were obtained in which the capillary endothelium stood out very clearly, the picture not being complicated by the staining of the connective-tissue cells.

Another method, necessitating only one perfusion, was also found useful. The perfusion fluid was suggested by the late Dr. M. B. R. Swann. Its composition was :—

Saturated alcoholic solution of basic fuchsin	17 c.c.
Absolute alcohol	63 c.c.
Glacial acetic acid	2 c.c.
Distilled water	18 c.c.

This solution was left in for 7 minutes and the vessels then washed through with saline. The whole preparation was finally immersed in 4 per cent. formaldehyde to fix the dye in the nuclei.

Control specimens were also made after the injection of 4 per cent. formaldehyde, followed by staining in watch-glasses by various methods. In this way the better selectivity of the injection-stained preparations could be appreciated.

Results of Observations by the Foregoing Methods.

The histological findings may be briefly summarized by commenting on the figures. All of these were made with the Abbé camera lucida.

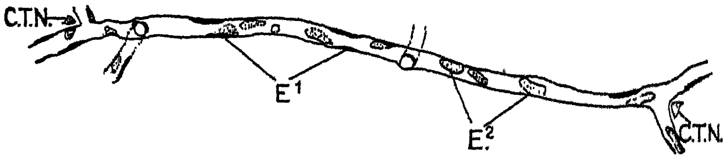

Fig. 1.—(× 320.) Capillary in mesentery of cat. Intra-arterial injection of both formol and stain. C.T.N. = nuclei of connective tissue cells; E^1 = endothelial nuclei in profile; E^2 = endothelial nuclei seen "en face."

Fig. 1 shows a piece of capillary some 305 μ in length. (There was a curve in this vessel about one-third along it, but the vessel has been straightened in the sketch for convenience in reproduction.) It is to be noted that the only elements, which could possibly be interpreted as Rouget cells, are certain nuclei depicted at CTN ; both these nuclei occur at branches of the vessel, and no other cells in relation to the vessel wall—apart from obvious endothelial nuclei—can be seen, in spite of the fair length of vessel. Further, neither of these nuclei shows any cytoplasm, even when examined with the highest powers available* (a 3-mm. apochromatic oil-immersion objective and a No. 18 compensating ocular). This is in contrast with the elaborate branching cytoplasm of the amphibian Rouget cells, as described by Vimtrup (5, 6). The absence of any resolvable cytoplasm, and the distance of these nuclei from the vessel wall, point to their being nuclei of connective-tissue cells lying in the ground substance of the mesentery.

Fig. 2 depicts a portion of the capillary network around fat cells—the latter not shown. Here again it is impossible to find cells suggestive of Rouget cells. Apart from the obvious endothelial nuclei, there are only two elements calling for comment. One of them is a slightly lobed nucleus shown at X ; no cytoplasm can be discerned, and its position and appearance stamp it as an endothelial nucleus of rather unusual shape. The other element is shown at CT ; it is a large cell, lying only partly in contact with the capillary wall. It has none of the characteristics attributed to Rouget cells, and we feel justified in regarding it as a connective-tissue cell, probably of the wandering type.

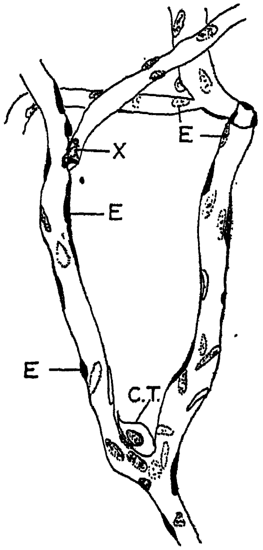

Fig. 2.—(× 360.) Capillary network round adipose tissue cells in mesentery of dog. Intra-arterial injection of both formol and carbol-fuchsin E = endothelial nuclei seen in profile and "en face"; C.T. = connective tissue cell; X = endothelial nucleus of rather unusual shape.

Fig. 3 shows a capillary after an intra-arterial injection of 4 per cent. formaldehyde followed by staining in a watch-glass.

Two features especially distinguish a preparation so made from the injection-fixed *and* injection-stained specimens. First, the capillary outlines are far less clear ; the walls are unstained and often difficult to distinguish from the surrounding tissues. Secondly, the connective-tissue cells and nuclei are widely and intensively stained. In the figure the latter are shown at CTN ; close to the vessel are three mast cells (M). Sometimes, as at X, the connective-tissue nuclei lie fairly close to the vessel wall. But there is a space between such nuclei and the capillary, nor can the branching processes of the amphibian Rouget

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* More powerful objectives (*i.e.* those of a shorter focal distance) are not suitable owing to the thickness of the mesentery.

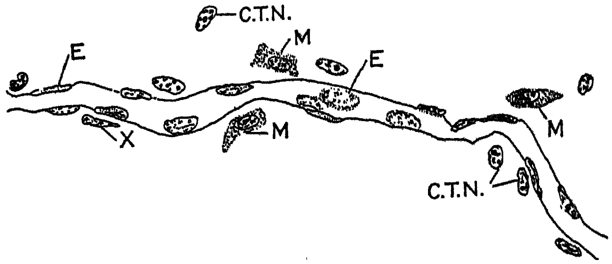

Fig. 3.—(× 500.) Capillary in mesentery of cat. Intra-arterial injection of formol followed by staining in bulk in a watch-glass with basic fuchsin. E = endothelial nuclei seen in profile and "en face"; C.T.N. = connective tissue nuclei; X = a connective tissue nucleus lying quite close to the capillary wall; M = mast cells.

cells be made out. Furthermore, such nuclei and cells grade into the free-lying connective-tissue cells of the mesentery.

In fig. 4 is depicted another portion of capillary after treatment similar to fig. 3. Here again it is impossible to see morphologically definable Rouget cells. Nuclei

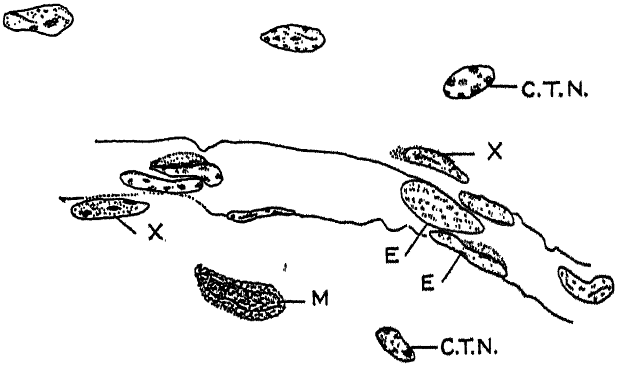

Fig. 4.—(× 900). Capillary in mesentery of cat. Intra-arterial injection of formol followed by staining in bulk with basic fuchsin in a watch-glass. E = endothelial cells seen in profile and "en face"; C.T.N. = connective tissue nuclei; X = connective tissue cells and nuclei lying close to the vessel wall; M = a mast cell.

of connective-tissue cells (CTN) are plentiful around the vessel. Sometimes, as at X, they lie quite close to it; but always is there a space between them and the capillary wall, nor can the characteristic processes be seen.

Intra-arterial injections of silver nitrate, followed by perfusion with Swann's fluid, were also made. But although the outlines of the endothelial cells were very clearly demonstrated, nothing resembling the outlines of Rouget cells was ever made out.

Discussion.

These observations on the staining of capillaries by intra-arterial fixation and staining have led us to conclude as follows :—

The number of elements present which could conceivably be regarded as Rouget cells (granting them to be contractile) seems to be quite insufficient to account for capillary contractility. Morphologically definable Rouget cells, in the sense of specialized contractile cells closely applied to the capillary wall, cannot be seen in our material, and this in spite of the method of intra-arterial fixation and intra-arterial staining—a method which, in our hands, has given better pictures of the vascular tree than the technique of staining isolated pieces of mesentery in watch-glasses.

While it is true that cells and nuclei are often found in close proximity to the capillaries, such elements have none of the features of Rouget cells, and appear rather to be connective-tissue elements of various types. Furthermore, there is nearly always a definite space between such elements and the capillary wall. That this space is an artefact, due to shrinkage after fixation and dehydration, we are inclined to doubt, since it is consistently found in specimens which show no other signs of shrinkage.

Our conclusions tally with those of E. R. and E. L. Clark (2, 3) in regard to the Rouget cells of amphibian larvæ. These authors studied the capillaries in the tail of narcotized Amphibian larvæ. They noted that the Rouget cells were derived from young connective-tissue cells, which they observed to migrate towards the capillary walls, and there to spread out in the form described by Vimtrup. The Clarks also noted independent contractions of the capillaries over areas devoid of Rouget cells. And, finally, they observed segments of capillaries to contract away from the Rouget cells, thereby leaving a space—as noted by us above—between the vessel and the alleged contractile cell.

Kiyono, whose researches have only been available to us through the account of them in Aschoff's 'Lectures on Pathology,' is also of opinion that capillary contractility in mammals is not a function of the Rouget cells. The latter he regards as belonging to the reticulo-endothelial system of Aschoff, who likewise is in agreement with this view (1).

Lest it should be thought that Rouget cells would not be demonstrated by the methods we used, we would point out:—(1) that stains such as Heidenhain's iron hæmatoxylin are amongst the most precise known for demonstrating both nucleus and cytoplasm; (2) even assuming that the cytoplasm of Rouget cells was not stained in our preparations, nuclei sufficiently close to the vessel wall and in sufficient numbers could not be found. We know of no evidence to show that the Rouget cell nucleus is difficult to stain; indeed, nuclei wherever they occur respond to the commonest stains. The neuroglia nucleus, so easily stained, and the neuroglial cell body and processes, necessitating such special methods, illustrate this point.

II. In Vivo Observations.*

From our morphological studies it would seem that the capacity of the capillaries to contract cannot be ascribed to Rouget cells. Even so, the possibility would still exist that the capillaries studied were not capable of contraction. For the purpose of investigating the reactions of the vessels an appropriate technique had to be evolved.

Technique.

A large deep tin was insulated by surrounding it with sawdust enclosed in an outer wooden box. A sloping board, the tilt of which could be varied, was placed across the back half of the box. A cat, narcotized with chloral, was slung from this board in a folded duster. The tin was filled with normal saline, which was kept at 38° C. A loop of gut was then extracted through a longitudinal incision in the posterior abdominal wall just above the spinal muscles of the right side. This operation was performed beneath the saline. The loop of intestine was next spread by fastening it by threads through the muscular coat to a glass circle supported in a clamp at a suitable height.

The mode of lighting the mesentery so as to observe it with a binocular dissecting microscope was due to a suggestion of Sir Charles Sherrington. A total reflection rod was used, pieces of ground glass being cemented on to each end of the rod so as to get a more even diffusion of the light, which was supplied by a 100-c.p. Pointolite lamp. A green glass screen was used, and it was possible to move about the short end of the reflection rod beneath any part of the mesentery which was to be examined. By means of this apparatus it was possible to see the mesenteric circulation under approximately normal conditions.

The capillaries in the mesenteric membrane itself are rather sparse, but, when occurring, are long. The places in which they are the most easily studied are near the fat deposits.

In preparations such as these, the " bead " phenomenon of corpuscular flow

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* For these observations one of us (H. W. F.) is responsible.

—already described by one of us (H. W. F.) in the cerebral circulation (4)—was seen very clearly.

The Effects of Mechanical Stimuli.

A finely pointed glass rod was used for this purpose. By gentle manipulation it was relatively easy to produce a dilatation, not only confined to the place stimulated but extending some distance on either side of the point stimulated. This may be due to the inexact localization of the stimulus as the mesentery suffers movement due to the peristalsis of the gut. It was, however, found very difficult to make a capillary contract with mechanical stimulation. Occasionally it was seen after very vigorous stimulation, but far more often the vessel became so damaged that it passed into a condition of stasis. The same effects were noted with weak electrical stimulation, a unipolar electrode being used.

Stimulation by electrical and mechanical means having yielded somewhat unsatisfactory evidence of motor activity of the capillaries, the effects of drugs were studied. In these observations, attention was mainly devoted to securing ocular evidence that the capillaries were capable of independent motor activity.

Effects of Drugs.

Histamine.—It was thought that this preparation afforded an excellent opportunity for observing the effect of histamine on these vessels. Rich had previously tried to utilize the omentum for this purpose, but he placed the omentum in air and was confronted with a permanent dilatation of all the vessels. By the method here described this was not the case.

The injection of a suitable dose of histamine into the saphenous vein of the cat was followed by the appearance of a large number of capillaries not previously noticeable, and those which had been visible before the injection now became much plainer and obviously carried a much greater blood flow. At the same time it was observed that the small veins now contained a much more concentrated blood, the corpuscles being very closely packed together. The veins, however, did not exhibit any contraction ; on the contrary, they were dilated. The arterioles were somewhat, though not markedly, contracted.

It would seem that the statement that the histamine effects are due to a contraction of the small venules will not suffice to explain the capillary effects of the drug.

An interesting effect of this substance was, however, noted when endeavouring to stain the endothelial cells by the method described above. As soon as the mesentery and gut were removed from an animal previously given histamine, all the large mesenteric veins suffered an extreme contraction. The blood was forced into pockets, so that in some cases the vein resembled a string of sausages with long intervals between them. This venous effect has been described by Dale, but he states that in an intact animal it took some considerable time to occur. In these experiments it occurred as soon as three minutes after the injection of the drug and immediately on removal of the mesentery. In consequence of this extreme contraction it was very difficult to wash the blood from the vessels by perfusion with saline; in fact, in no single case was it possible to free the gut entirely from blood with a perfusion of as long as 10 minutes, whereas a normal gut is clear in a minute or two.

Pituitrin.—The effect of this drug was tried both by local application and intravenous injection. For local application the pituitrin (17 per cent.) was taken into a pipette with a fine opening. The pituitrin was then ejected against the piece of mesentery under observation. As the injection was necessarily made into the saline of the bath the pituitrin was rapidly diluted.

With these conditions it could be seen that this drug caused a rapid and complete closure of all the capillaries of the area. The arterioles were not greatly affected, but the blood stream in the veins became much slowed and showed marked clumping of the corpuscles. That this was due to the fact that the capillaries had closed down actively was shown by the fact that as these vessels recovered and again dilated the blood stream in the veins was restored to its previous dimensions.

On injection into the blood stream the same disappearance of capillaries was noted. In this case, however, the arterioles suffered a greater diminution in their calibre than when the drug was applied locally.

After a histamine injection, when all the capillaries were widely dilated, the application of pituitrin was still just as efficacious in causing the capillaries to contract. This is some support for the view already put forward, that the beneficial effects of pituitrin in surgical shock are due to its constrictive effects on the capillaries.

Adrenalin.—The local application of adrenalin in the same way was followed by a contraction of the arterioles but no contraction of the capillaries; on the contrary, there appeared to be a dilatation, in some cases followed by a very prompt stasis.

If the adrenalin were injected intravenously, it was then observed that accompanying the arterial constriction was a very marked increase in the rate of the blood flow through the capillaries, but these vessels were not affected in their calibre.

Summary.

From these experiments it would appear that the mesenteric capillaries are capable both of contracting and expanding in an active way.

That the presence of Rouget cells in sufficient numbers to account for the contraction is doubtful, has been indicated by the histological studies, so that one is forced to the conclusion that in Mammalia (as typified by the cat) the motor activity of the capillaries resides in the endothelial cells.

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The Fate of the Sugar Disappearing under the Action of Insulin.

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The apparent disappearance of sugar injected into normal animals has for a long time puzzled physiological investigators (Bang, Meltzer and Kleiner, Palmer, Woodyatt). When insulin was discovered it was apparent that an agent was available by which the normal processes could be exaggerated, and therefore more easily studied. It was soon shown that the administration of sugar and insulin to the diabetic animal resulted in an increased combustion of carbohydrate and the accumulation of glycogen in the depôts. When, however, attempts were made to trace the fate of the sugar which disappears from the blood of the normal animal under the influence of an injection of insulin, difficulties were encountered.

McCormick and Macleod (1) studied the effect of insulin on the glycogen reserves of rabbits which had been starved and treated with epinephrin. In some of the experiments glucose was administered subcutaneously during the period of action of insulin. No significant difference between the glycogen content of the muscles of the control animals and of those which received insulin was observed. The glycogen of the livers of the insulin-treated animals was slightly less than that of the control animals. Macleod (2) concluded from these experiments "that less glycogen is deposited both in the muscles and the liver when insulin is given along with sugar to previously starved animals than when the same amounts of sugar are given alone." In the experiments of Dudley and Marrian (3), in which the effect of insulin on the liver glycogen of mice was studied, a much smaller amount of glycogen was found in the livers of the animals which received insulin than in those which served for controls. In another series of experiments in which insulin was administered to rabbits which had been previously fed on a carbohydrate rich diet, the glycogen content of the liver and skeletal muscles of the insulin-treated animals was again much less than that of the control animals. In both series of experiments the animals were killed after convulsions had supervened. The experiments of Babkin (4) are similar to those of McCormick and Macleod. In some of his experiments Babkin kept the blood sugar of the rabbits at a high level by the administration of sugar. He found no increase in glycogen after insulin. Kuhn and Baur (5), in a study of the effect of insulin on the glycogen content of the skeletal muscles of rabbits and guinea-pigs, found that, after insulin convulsions, the glycogen had practically disappeared from the muscles of these animals. They are undecided as to whether the depletion of glycogen is a primary effect of insulin or is to be attributed to the convulsions.

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Other investigators believe that the glycogen content of the animal body is increased under the action of insulin. Bickel and Collazo (6) found that the glycogen content of the livers of pigeons, fed on a diet deficient in vitamin B, was increased in those which received insulin. They obtained similar (7), but not very conclusive, results in normal guinea-pigs. Convulsions were carefully avoided in these experiments. Bissinger, Lesser and Zipf (8), in a study of the effect of insulin on the carbohydrate balance of mice, determined the glycogen content of starved mice, of mice injected with dextrose only, and of mice which received dextrose and insulin. They found that approximately one-third of the sugar which disappeared could be recovered as glycogen. In a more recent paper Lesser and Bissinger (9) have shown that one-half hour after the injection of insulin about one-fifth of the sugar is deposited as glycogen. Cori, Cori, and Pucher (10) have reported that insulin causes an increase in the liver glycogen of normal rabbits when the free sugar of the liver is as low as in a starving animal. Ringer (11), using phloridzinized dogs, found that 40 per cent. of the sugar which was metabolized by these animals as the result of an injection of insulin was burned. He assumed that the remainder was laid down as glycogen. Cori (12) has shown that there is an increase in the glycogen of the liver of phloridzinized rabbits and cats when insulin is administered. He has found (13), however, that the liver glycogen of mice is decreased by insulin, even when convulsions are avoided.

The conclusion, that the sugar which disappears from normal animals under the action of insulin is not laid down as glycogen, has been accepted by many investigators, and numerous theories have been advanced to explain this disappearance. One of the most interesting hypotheses is that there is an increase in lactacidogen (Emden) sufficient to account for a large part of the sugar. Harrop and Benedict (14), using unanæsthetized animals, compared the lactacidogen content of a muscle removed before insulin with that of the corresponding muscle of the opposite side removed some time after insulin. They believe that they have demonstrated an increase in lactacidogen after insulin. (See also Audova and Wagner (15).) Kay and Robison (16), who have reviewed the literature which deals with the relation of phosphorus to carbohydrate metabolism, also obtained results which convinced them that the lactacidogen of the muscle is increased under the action of insulin. Eadie, Macleod, and Noble (17) have been unable to confirm these findings. Many of the other theories which have been advanced to explain the rapid disappearance of the sugar from the blood have been disproved, while others, which might account for a stage in this process, could not be expected, nor were some of them intended, to explain the ultimate fate of the sugar.

The experiments of Burn and Dale (18) on the eviscerated spinal cat show that the increase in oxygen consumption after the administration of insulin is usually insufficient to account for more than a small part of the sugar which disappears from the blood of this preparation. Our experiments, which may be regarded as an extension of the work of these investigators, are designed to investigate the fate of this sugar which is not burned. Attention was first concentrated on lactacidogen. The change in muscle glycogen attributable to insulin was then investigated.

Methods.

Choice of a preparation.—In many of the experiments which have been mentioned above a series of animals have been used as controls for a second series to which insulin has been administered. The glycogen content of the tissues of the control animals, however, varies within such wide limits that an almost prohibitive number of animals must be used before valid conclusions as to the effect of insulin can be drawn. In studies in which normal rabbits or mice have been used convulsions have frequently occurred in spite of the administration of dextrose. Furthermore, in many experiments in which the intact animal is used, it is not certain that sufficient sugar disappears from the blood, in excess of what may be burned or excreted, to give a detectable increase in glycogen, if this substance were formed. Variations in the interval between the cessation of circulation with the death of the animal and the fixation of the tissues for estimation of this glycogen are difficult to eliminate in experiments on the intact animal. The eviscerated spinal preparation (Burn and Dale) is particularly well adapted to an investigation of this kind. Dextrose is infused at any desired rate into the jugular vein by means of the slow infusion apparatus of these authors. The blood sugar can be kept approximately at any desired level. Convulsions are avoided. The glycogen content of an individual muscle of one leg has been shown to be practically the same as the content of the corresponding muscle of the opposite leg (19). We have taken advantage of this fact to determine the effect of insulin on the glycogen content of the muscles of this preparation. To obtain the muscles, the skin is removed and each muscle in turn is dissected free from its sheath. Ligatures are placed around the extremities of the muscle. When everything is ready the ligatures are tied; the muscle is removed and immediately dropped into hot alkali.

Methods used in the analysis of tissues.—The blood sugar was estimated in the early experiments by the Shaffer-Hartmann method. In the later experiments the Hagedorn-Jensen technique was used. The latter method is much the more suitable for use with the eviscerated spinal preparation, since the small samples of blood required (0·2 to 0·3 c.c.) can be taken frequently without affecting the blood pressure. Inorganic phosphorus was estimated by the Briggs modification of the Bell-Doisy method. Glycogen was estimated by Pflüger's method. The sugar formed by the acid hydrolysis of the glycogen was determined by a modification of the Bertrand method. Five c.c. amounts of the sugar solution, copper reagent and alkali were used, instead of 20 c.c. as recommended in Bertrand's paper. The solutions were heated together for ten minutes in the boiling water-bath, instead of being boiled for three minutes over the flame. Bertrand's permanganate solution was diluted five times. The use of smaller quantities of sugar solutions made duplicate determinations possible. Emden's technique, except that trichloracetic acid was used instead of Schenck's reagent to precipitate the proteins, was followed in the lactacidogen determinations.

EXPERIMENTAL RESULTS.

I. *Phosphate Metabolism.*

Inorganic Phosphorus.—The decrease produced by insulin in the inorganic phosphorus of the blood of normal animals has been observed by many investigators. In the first series of experiments reported in this paper the effect of insulin on the inorganic phosphorus of the blood of the eviscerated spinal preparation has been studied.

Control Experiments.

Experiment 1.—Cat, 2·5 kgm. Eviscerated spinal preparation. Sugar infused at an average rate of 340 mgm. per hour.

Time	—	1 hr.	2 hrs.
Blood sugar percentage	0·292	0·294	0·300
Inorganic phosphorus of whole blood in mgm. per 100 c.c.	5·8	5·5	5·4

Experiment 2.—Cat, 2·6 kgm. Eviscerated spinal preparation. Sugar infused at an average rate of 200 mgm. per hour.

Time	—	30 min.	2 hrs.
Blood sugar percentage	0·352	0·360	0·300
Inorganic phosphorus of whole blood in mgm. per 100 c.c.	8·4	8·2	8·5

Experiment 3.—Cat, 2·7 kgm. Eviscerated spinal preparation. Sugar infused at an average rate of 180 mgm. per hour.

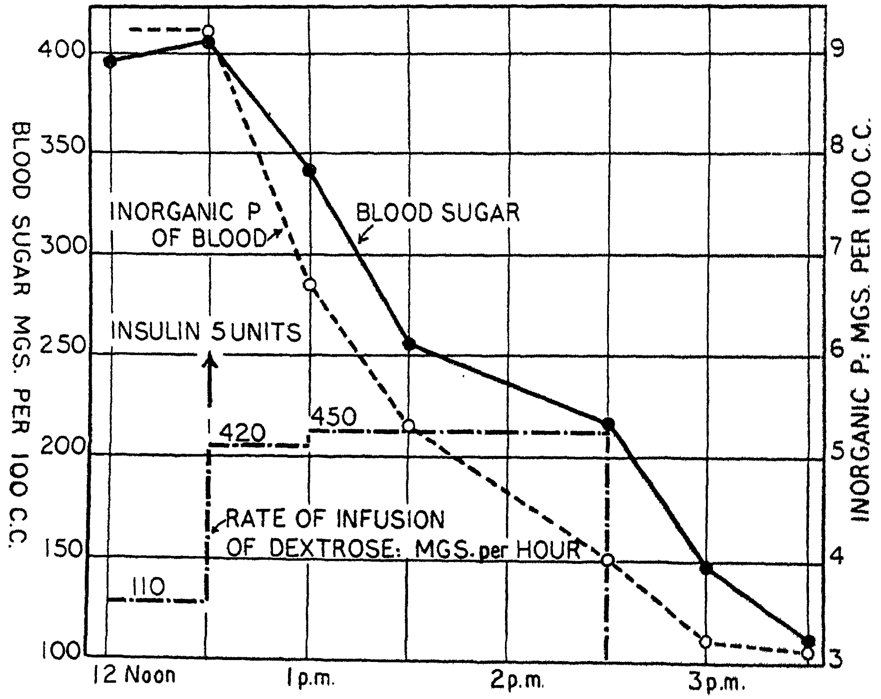
Time	—	1 hr. 15 min.	2 hrs.	2 hrs. 30 min.
Blood sugar percentage	0·273	0·267	0·235	0·203
Inorganic phosphorus of whole blood in mgm. per 100 c.c.	9·5	9·4	9·6	9·1

These experiments show that the inorganic phosphorus of the blood of the eviscerated spinal preparation remains fairly steady when the blood sugar level is kept constant by the infusion of sugar.

Insulin Experiments.

Experiment 4.—Cat, 3·7 kgm. Eviscerated spinal preparation. Sugar infused at the rate of 150 mgm. per hour.

Time	—	45 min.	55 min.	1 hr. 20 min.	2 hrs. 50 min.	3 hrs. 10 min.
Blood sugar percentage ...	0·237	0·242	Insulin	0·166	0·063	0·053
Inorganic phosphorus of whole blood	6·3	6·4	5 units	5·3	4·2	3·9



Experiment 5.—The details of this experiment are shown in fig. 1. The rate of sugar infusion was increased slightly after the administration of insulin so as to diminish the rate and extent of fall of the blood sugar.

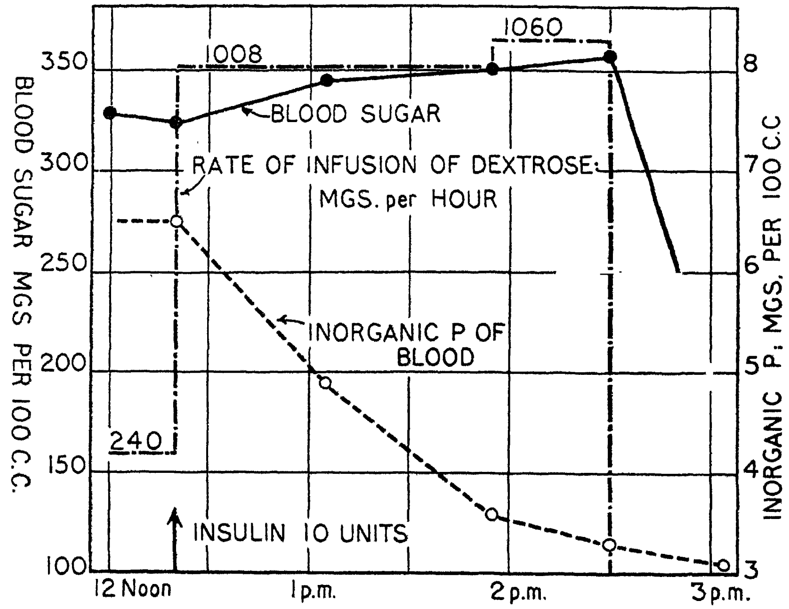


FIG. 2.

Experiment 6 (fig. 2).—In this experiment the rate of sugar infusion was increased when insulin was given and there was no fall in blood sugar.

These experiments show that insulin produces a decrease in the inorganic phosphorus of the blood of the eviscerated spinal preparation, and that when the blood sugar is prevented from falling by accelerating the infusion of dextrose, the fall in blood phosphate persists. The change in blood phosphate is, therefore, related to the disappearance of sugar and not to the fall in sugar concentration in the blood. In this preparation it is improbable that the blood receives phosphorus from any source, and we may assume that the phosphorus disappearing from the blood goes to the muscles. The effect of this additional inorganic phosphorus on the concentration of this substance in the muscles may be seen from the following calculation. The muscles of a cat weighing 3·0 kgm. weigh about 1·5 kgm. (Table 8). The active volume of the body fluids of the eviscerated spinal preparation made from this animal may be estimated as 500 c.c. (Burn and Dale). This "active volume" has been used in calculations throughout this paper. If the inorganic phosphorus of the blood decreases by 6 mgm. per 100 c.c., 30 mgm. of phosphorus have been distributed to the muscles, if our assumptions are correct. 30 mgm. of phosphorus spread over 1,500 gm. of muscle would give an increase of inorganic phosphorus of approximately 2 mgm. per 100 gm. of muscle. The change would be within the range of error of the methods used in the estimation of the phosphorus. This calculation takes no account of the effect which variations in the other forms of phosphorus of the blood might have on the inorganic portion. There appears, however, to be very little change in these other compounds (Kay and Robison).

Lactacidogen.—The effect of insulin on the lactacidogen content of the muscles was observed also by direct experiments. In Table I two types of control experiments are recorded, and a series of experiments in which insulin was administered. In the first experiment (1) the muscle from the right leg was removed and the corresponding one of the left leg immediately afterwards. In the second series of experiments (2) the blood sugar of the preparation was kept constant by the infusion of sugar. The right muscle was removed at the beginning of the experiment and the left one two hours later. In the third series (3), also carried out with infusion of dextrose, the right muscle was taken, insulin was injected, and the left muscle was removed from two to three hours later. In this third series of experiments the sugar disappearance varied between 3 and 8 grams in the period after insulin.

In Table I, A is the value for free inorganic phosphorus obtained when the muscle, immediately after its removal, is ground up with sand in ice-cold 2 per cent. trichloracetic acid. Value B represents the inorganic phosphorus after the muscle has been digested, at 37° C., for 18 hours in 0·25 per cent. sodium bicarbonate. A little chloroform is added to the mixture. Samples were taken after 2, 18, and 36 hours of digestion. The proteins were precipitated with 20 per cent. trichloracetic acid and the inorganic phosphorus estimated. Value B, after 18 hours, was usually from 2 to 4 mgm. per 100 gm. greater than after two hours. The difference between A and B gives the amount of lactacidogen (Emden). The free phosphorus figure in all the experiments is higher at the end than at the beginning. The fact that the circulation becomes less efficient as the experiment progresses may have a bearing on this finding. In the last column of the table the difference between the B values of the muscles of the two sides are given. The slight increase would account for the phosphate which disappears from the blood.

The figures appear to indicate that there is a slight increase in that part of the combined phosphoric acid which is set free relatively early in the digestion —in other words, in the lactacidogen of Embden. The differences, however, are so small that even their reality is doubtful; and, in any case, we can safely

Table I.—Inorganic Phosphorus and Lactacidogen in Skeletal Muscle.

		P. in mgm. per 100 gm. muscle.					
		Right leg.		Left leg.		Difference in Lactacidogen between Right and Left Side.	Difference between B values of Right and Left Side.
1. Control	A	87	—	88·5	—	—	—
	B	145	—	149	—	—	+ 4
Lactacidogen		—	58	—	60·5	+ 2·5	—
2. Control	A	101	—	100	—	—	—
Sugar infused	B	130	—	138	—	—	+ 8
Lactacidogen		—	29	—	38	+ 9	—
	A	86	—	93	—	—	—
	B	128	—	136	—	—	+ 8
Lactacidogen		—	42	—	43	+ 1	—
	A	88	—	93	—	—	—
	B	168	—	173	—	—	+ 5
Lactacidogen		—	80	—	80	0	—
3. Insulin experiments	A	82	—	91	—	—	—
	B	139	—	160	—	—	+21
Lactacidogen		—	57	—	69	+12	—
	A	85	—	90	—	—	—
	B	147	—	158	—	—	+11
Lactacidogen		—	62	—	68	+ 6	—
	A	88	—	93	—	—	—
	B	168	—	173	—	—	+ 5
Lactacidogen		—	80	—	80	0	—
	A	88	—	98	—	—	—
	B	139	—	153	—	—	+14
Lactacidogen		—	51	—	55	+ 4	—
Expt. 16	A	91	—	100	—	—	—
	B	147	—	150	—	—	+ 3
Lactacidogen		*	56	*	50	− 6	—
	A	97	—	102	—	—	—
	B	156	—	165	—	—	+ 9
Lactacidogen		†	59	†	63	+ 4	—
Expt. 17	A	91	—	97·5	—	—	—
	B	141·5	—	147	—	—	+ 5·5
Lactacidogen		*	50·5	*	49·5	− 1	—
	A	96	—	102	—	—	—
	B	156	—	162	—	—	+ 6
Lactacidogen		†	60	†	60	0	—

conclude that no significant proportion of the sugar which has disappeared is stored as a phosphoric ester. It has been shown above that the disappearance of sugar from the circulation of the eviscerated spinal preparation is associated with a disappearance of free phosphate, and this suggests that phosphoric esters play a part in the phenomenon. It is now clear, however, that they can only function as a stage on the way to the true storage form.

II. *Glycogen.*

The second part of our investigation consists in a study of the variation in the glycogen content of the skeletal muscles when insulin and sugar are administered to the eviscerated spinal preparation. A series of control experiments are recorded first. The great variation in the glycogen content of the different muscles of the same animal, and in similar muscles of different animals, is well demonstrated. The contents of the corresponding muscles of the two hind limbs, however, agree rather closely under the conditions of these control experiments. Although in some cases there is a difference of 20 per cent. between the glycogen contents of two corresponding muscles, the difference between the average values for several corresponding muscles of the two sides is small. The small differences between the glycogen figures for corresponding muscles are probably due, in part, to the unavoidable variations in the technique of removal.

1. *Control Experiments.*

Experiment 7.—I. Cat, 2·7 kgm. Eviscerated spinal preparation. Muscles of right side taken first, left side 20 min. later.

Table II.—Glycogen.

	R.	L.
	Per cent.	Per cent.
Gastroc.	0·508	0·474
Tibialis	0·338	0·309
Sartorius	0·266	0·241
Triceps	0·340	0·291
Rectus Femoris	0·538	0·540
	Av. 0·398	Av. 0·371

Experiment 8.—II. Cat, 3 kgm. Eviscerated spinal preparation. Muscles of right side taken first, left side 20 min. later.

Table III.—Glycogen.

	R.	L.
	Per cent.	Per cent.
Gastroc.	0·337	0·385
Tibialis	0·338	0·421
Sartorius	0·433	0·401
Biceps	0·302	0·325
Rectus Femoris	0·479	0·440
	Av. 0·378	Av. 0·394

In Experiments 9, 10 and 11 muscles were taken in the same way from eviscerated spinal cats subjected to constant intravenous infusion of dextrose, at rates of 400–500 mgm. per hour. The muscles of the right leg were taken early in each experiment, those of the left leg after 1 hr. 15 min., 2 hrs. 30 min., and 5 hours respectively in the three experiments. The percentage of dextrose in the blood rose from 0·276 to 0·302 in Experiment 9, from 0·354 to 0·398 in Experiment 10, and from 0·294 to 0·430 in Experiment 11, during these periods. The muscles were therefore exposed to progressive hyperglycæmia of 0·3 to 0·4 per cent. for these different periods, and furnish good controls for those from the later experiments, in which sugar was given with insulin. The averages of their glycogen contents were as follows :—

Table IV.

	R.	L.	Difference.
Experiment 9	0·257	0·261	0·004
,, 10	0·589	0·598	0·009
,, 11	0·388	0·419	0·031

It will be seen that the maximum difference, 0·031 per cent., is so small as to have doubtful significance. The maximum difference between individual muscles was 0·086 per cent. These experiments confirm the statements of Külz (19), of Hatcher and Wolf (20), and of others, that isolated mammalian muscles do not form glycogen when perfused with blood rich in dextrose.

2. *Insulin Experiments.*

In Experiments 12–17 insulin was administered and the rate of sugar infusion was increased in an attempt to maintain a hyperglycæmia comparable to that which was present in the control experiments. The initial dose of insulin in these experiments was 12 units. This dose was repeated at the end of each 90 minutes during the "insulin period."

Experiment 12.—Cat, 3·4 kgm. Eviscerated spinal preparation.

Before injection of insulin: Dextrose infused at a rate of 480 mgm. per hour. Initial blood sugar, 0·308 per cent.; after 45 minutes, 0·287 per cent.

After the injection of insulin: Dextrose infused at a rate of 1,476 mgm. per hour. After 2 hrs. 40 min. the total amount of dextrose infused was 3·924 gm. The blood sugar decreased from 0·287 per cent. to 0·178 per cent.—that is, 0·109 per cent.

The calculated "active volume" of this cat would be 565 c.c. There was, therefore, a disappearance of 109 × 5·65 = 616 mgm. sugar in addition to the dextrose infused. Therefore the total sugar diappearance was 4·54 gm., on this basis of calculation.*

The muscles of the right side were taken before insulin was given, those of the left side 2 hrs. 40 min. after the insulin injection.

The glycogen contents, expressed in percentages of the weights of the fresh muscle, are shown in Table VII.

Table V.—Glycogen.

	R.	L.
	Per cent.	Per cent.
Tibialis (ant.)	0·602	0·714
Sartorius	0·673	0·767
Rectus Femoris	0·485	0·617
	1·760	2·098
	Av. 0·587	Av. 0·699
	Increase	0·112 per cent.

The muscles of a cat represent approximately 50 per cent. of the weight of the cat, as shown by the figures in Table VI.

Table VI.—Weight of muscles in relation to body weight.

Cat, weight	3·4 kgm.	3·2 kgm.	2·9 kgm.
Muscles + bones, without head	2·053	1·920	1·821
Bones	0·403	0·320	0·260
	1·650	1·600	1·561
M. taken for experiment....	0·040	0·040	0·030
Total weight of muscles	1·690	1·640	1·591
Percentage of body weight	50	51	54

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* In our more recent experiments we have found that several important sources of carbohydrate are not considered in these estimates. It has been found that, although the vessels carrying blood to the liver are tied, an appreciable quantity of dextrose enters the blood from this source during the experiment. Therefore more sugar is used than our calculations indicate, and the figure for sugar disappearance is too low.

If we assume that the increase of glycogen found in the muscles of the hind limb is representative of that in all the muscles of the body, the total increase in muscle glycogen may be estimated

$$0\cdot112 \times 17 = 1\cdot9 \text{ gm.}$$

If now we make the balance

Total dextrose lost	4·5 gm.
„ „ found as glycogen	1·9 „
Missing	2·6* „

Experiments 13 and 15, which lasted for approximately the same period, and in which the calculated sugar disappearance was about equal, are not comparable in other respects. In Experiment 13 the blood sugar level was 0·43 per cent., and this high level was maintained for 3 hours 30 min. After this it fell, and at the end of the experiment was 0·20 per cent. In Experiment 15 the blood sugar at the beginning was at 0·251 per cent. After 2 hours 15 min. it was at 0·131 per cent., and remained at that level for the following 3½ hours. There may be a relationship between the level of the blood sugar and the proportion of sugar laid down as glycogen, but this has not been directly investigated.

In Experiments 16 and 17 we tested the possibility that an increase in the lactacidogen might take place during the first period of insulin action, with only a slight increase in glycogen, and that, later, a larger proportion of sugar might be laid down as glycogen. This experiment was carried out as follows:—

Four muscles of the right side were taken before insulin was given. Glycogen estimations were made in two of these, and the inorganic phosphorus, before and after digestion, was estimated in the two others. After 1 hour 40 min. of insulin action, two muscles of the left side were taken, one for glycogen and one for phosphorus estimation. At the end of the experiment the two other muscles of the left side were taken.

The lactacidogen analyses are given in Table I. The values for the pairs of muscles marked with (*) in the table show the difference produced in 1 hr. 40 min., those marked with (†) the difference produced in 3 hours or more of insulin

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* In some more recent experiments the oxygen consumption has also been measured. The figures show that the sugar which disappears, and is not deposited as glycogen, is fully accounted for by the oxygen consumption, as will be shown in a forthcoming paper.

Experiments 13, 14 and 15 were on similar lines, and their results may be tabulated.

Table VII.

	Wt. of cat.	Insulin period.	Blood sugar percentage.		Total dextrose infused.	Calc. loss of dextrose.	Muscle glycogen percentage.			Calc. total gain of glycogen.
			Before insulin.	Final.			Before insulin.	Final.	Increase.	
Expt. 13	2·6	5 hrs. 30 min.	0·430	0·200	7·278 g.	**8·3** g.	0·499	0·807	0·358	**4·71**
Expt. 14	3·1	3 hrs. 40 min.	0·330	0·506	6·168 g.	**5·3** g.	0·543	0·730	0·187	**2·9**
Expt. 15	3·1	5 hrs. 45 min.	0·251	0·131	8·64 g.	**9·2** g.	1·162	1·342	0·180	**2·8**

action. It is clear that no definite increase occurred at any stage. The glycogen, however, increased, as shown in the following record of Experiment 16 :—

Experiment 16.—Cat, 2·19 kgm. Eviscerated spinal preparation.

Before insulin injection—50 min.—the blood sugar remained steady at 0·382 per cent.

Dextrose was infused at a rate of 454 mgm. per hour.

After the injection of insulin :

1. First period of 1 hr. 40 min. Blood sugar steady at 0·382 per cent. Dextrose had been infused at a rate of 1,369 mgm. per hour.

Table VIII.—Glycogen.

	R. Before insulin.	L. 1 hr. 40 min. after insulin.
Tibialis	0·512 per cent.	0·723 per cent.
	Increase—0·211 per cent.	

2. 3 hrs. 30 min. after insulin. Blood sugar had fallen from 0·382 to 0·251 per cent. Dextrose had been infused at a rate of 1,500 mgm. per hour.

Table IX.—Glycogen.

	R. Before insulin.	L. 3 hrs. 30 min. after insulin.
Sartorius	0·419 per cent.	0·717 per cent.
	Increase—0·298 per cent.	

Dextrose infused : 5·04 gm. Sugar disappeared from the blood : 0·131 × 5 = 0·655 gm. Total sugar disappearance, 5·7 grams.

Glycogen increase, 0·298 × 14·5 = 4·3 grams. This estimate, based only on the increase in one muscle, has of course less quantitative significance than those in the previous experiments. If calculation is made from the increase in one muscle after 1 hr. 40 min., a total gain of glycogen of about 3 grams is obtained, whereas only about 2·3 grams of sugar had been given.

The results of the foregoing insulin experiments are summarised in Table X.

Insulin Experiments.

Table X.

No. of experiment.	Duration. hrs. min.	Sugar disappearing.	Glycogen found.	Sugar not recovered as glycogen.*
12	2 40	4·5	1·9	2·6
13	5 30	8·3	4·7	3·6
14	3 40	5·3	2·9	2·4
15	5 45	9·2	2·8	6·4
16	3 30	5·7	4·3 (one muscle)	1·4
	Average (without Ex	pt. 16) 6·8	3·1	3·7

* See footnotes pp. 42 and 43.

The results show that, in the preparation used in these experiments, when plenty of dextrose is supplied, insulin will cause the deposition of a large proportion, about 45 per cent. on an average, as glycogen in the skeletal muscles.

The conditions of the experiments hitherto described practically limited the action of insulin to the muscles, as the only tissues remaining which had an important carbohydrate metabolism. It was thought desirable to make further experiments, using the intact animal, and securing immobilization by chemical anæsthesia in place of destruction of the brain. Among anæsthetics available for this purpose, that known as "Amytal" appeared, from the experience of others, to be the most desirable. We made a few experiments with "Amytal,"* but we found that even this anæsthetic introduced complications which rendered it unsuitable for our purpose. Under "Amytal" alone the muscles lost glycogen, and a dose of insulin, which reduced the blood sugar to 0·07 per cent., did not prevent this loss. In order, therefore, to include the liver in the range of observation, but to leave the conditions otherwise as in the previous experiments, we made a few experiments on the animal with the brain destroyed, but the abdominal viscera left intact (Experiments 18, 19, and 20).

In Experiment 18 no analysis of the liver was made. During the 4 hrs. and 15 min. of the insulin period, in which 30 units in all were given, 4·42 gm. of dextrose were infused, and the blood sugar fell from 0·387 to 0·282 per cent. The average percentage of muscle glycogen increased in the same period from 0·856 to 1·120, *i.e.*, by 0·264 per cent.

In Experiment 19, samples of liver as well as of the muscles were taken just before insulin was first administered, and again at the end of the experiment. In the 4 hours of insulin action 4·57 gm. of dextrose were infused, and the blood sugar fell from 0·440 to 0·220 per cent. The muscle glycogen increased from 0·930 to 1·142, *i.e.*, by 0·212 per cent., and the liver glycogen from 2·245 to 2·83, *i.e.*, by 0·585 per cent. Such an increase in the liver glycogen would contribute but little to the total gain of glycogen by the preparation, since the weight of the liver was probably not more than 1/15 of that of the muscles.

In Experiment 20, with the idea that the liver might take a greater part in the glycogen formation if the insulin reached it first, both dextrose and insulin were administered, in mixed solution, by infusion into the splenic vein, so that both passed through the liver before reaching the general circulation. The urine excreted in the insulin period was also collected and analysed. The action of insulin lasted for 3 hours, during which 3·036 grams of dextrose and

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* Kindly placed at our disposal by Messrs. Eli Lilly and Company.

25 units of insulin were infused. Two samples of liver were taken before insulin, and two at the end of the experiment. The blood sugar fell from 0·500 to 0·375 per cent. The urine secreted during the period contained 0·636 gm. of dextrose. The muscle glycogen increased from 0·935 to 1·297, *i.e.*, by 0·362 gm. of dextrose. The liver glycogen, however, fell from 6·86 to 2·63 per cent., *i.e.*, by 4·23 per cent., giving extra dextrose to be accounted for, instead of an addition to the glycogen yield.

Weight of cat	3 kgm.
Weight of liver	100 gm.
	gm.
Dextrose from liver	4·23
Dextrose infused	3·036
Blood sugar fell 0·125 per cent., 0·125 × 5	0·625
	7·891
Dextrose excreted in urine	0·636
„ to be accounted for	**7·255**
Glycogen increase in muscles, 0·362 × 15	**5·43**

	gm.
Sugar to be accounted for	7·891
„ excreted	0·636
„ disappearing	**7·255**
„ found as glycogen in the muscles, 0·362 × 14·8 =	**5·36**

It will be seen that the increase in liver glycogen in Experiment 19 was relatively very small, and that in Experiment 20, made with intention of concentrating the insulin effect on the liver as far as possible, there was actually a large loss of glycogen from that organ. It may be doubted, however, whether the conditions of the experiment entitle us to conclude that the liver, under completely physiological conditions, does not participate in the immediate formation of glycogen from glucose under insulin. The experiments are useful, however, as showing that the deposition in the muscles is not an artificial result of the exclusion of other organs. With the abdominal viscera intact, it occurs at least as rapidly as when they have been removed. As we were interested in the fate of the sugar, rather than the effect attributable to natural as distinguished from artificially injected insulin, experiments with infusion of dextrose alone, with intact viscera, were not made.

The Effect of Hypoglycæmia upon Muscle Glycogen.

It has been suggested by several investigators that when insulin is administered to an animal which has only a small amount of available carbohydrate, the glycogen of the muscle is given up to form some unknown sugar compound. Bollman, Mann and Magath (22) have recently shown, however, that in hepatectomised dogs the muscle glycogen is not drawn upon to maintain the normal blood sugar level. To investigate the effect of simple hypoglycæmia upon the muscle glycogen of the eviscerated spinal preparation, experiments were performed in which a small amount of sugar, or no sugar at all, was infused.

In Experiment 20, which lasted 3 hours, no dextrose was infused and no insulin was administered, so that the effect of simple hypoglycæmia was studied, due to exhaustion of the circulating dextrose.

Experiment 21.—Cat, 2·74 kgm. Eviscerated spinal preparation. No insulin or sugar. Initial blood sugar, 0·30. After 1 hr. 15 min., 0·22 per cent.; 3 hrs. 15 min., 0·132 per cent.; 5 hrs. 15 min., 0·068 per cent. There was no significant change in the glycogen content of the muscles. Those taken at the beginning of the experiment had an average content of 0·736 per cent.; those at the end of 5¼ hrs., 0·777 per cent.

In Experiments 22 and 23 insulin was administered and sugar infused throughout the experiment, but the rate of infusion was but little increased after insulin, so that the blood sugar fell, ultimately, well below the normal fasting level, as will be seen from the following figures.

Experiment 22.—Cat, 3·2 kgm. Eviscerated spinal preparation. Before injection of insulin dextrose was infused at a rate of 200 mgm. per hour; after the injection the rate was 265 mgm. per hour.

Time from start, in minutes.	—	45	70	120	200	205	270	320
Blood sugar ...	0·259 per cent.	0·278 per cent.	Insulin, 10 units.	0·232 per cent.	0·131 per cent.	Insulin, 10 units.	0·106 per cent.	0·066 per cent.

Right side muscles were taken after 45 min.
Left ,, ,, ,, at the end of experiment.

Experiment 23.—Cat, 3·725 kgm. Eviscerated spinal preparation. Before injection of insulin dextrose was infused at a rate of 184 mgm. per hour; after insulin the rate was 240 mgm. per hour.

Time from start, in minutes	—	120	Insulin,	200 min.	270 min.
Blood sugar ...	0·300 per cent.	0·270 per cent.	10 units.	0·115 per cent.	0·088 per cent.

Right side muscles were taken after 80 min.
Left ,, ,, ,, at the end.

In spite of the hypoglycæmia the muscle glycogen increased in Experiment 22 from an average of 0·854 per cent. to 1·020 per cent., an increase of 0·166 per cent. In Experiment 23 the average muscle glycogen before insulin was 0·855 per cent. and after insulin 0·986 per cent., giving an increase of 0·131 per cent.

When calculation was made, from the results of these experiments, of the relation between the total glycogen deposited and the total sugar lost, on the basis adopted in the earlier experiments, we found that more glycogen had actually been formed than the loss of sugar, thus estimated, could provide. This led to a more detailed examination of the sugar which might trickle into the vena cava from the liver, and the loss of free sugar from the muscles, in addition to that from the calculated " active volume " of the circulation. In a later paper we shall deal with this more fully, and give figures for oxygen consumption as well. The point with which we are here concerned is that, in the spinal preparation, with its complete immobilization and low metabolism demand, there is no secondary depletion of the muscle glycogen under insulin, even when the latter is given in such excess as to reduce the blood sugar to a low value.

In Experiments 24 and 25 the effect of prolonged and severe hypoglycæmia on the spinal preparation was investigated, in the spinal preparation, with and without the abdominal viscera. No sugar was given from outside, except such small quantities as were needed to suppress convulsions which appeared at intervals in both cases, the amount so administered in Experiment 24 being altogether 100 mgm., in Experiment 25, 240 mgm. Olmsted and Taylor (21) have also reported the occurrence of muscle spasms in the spinal animal, which they believe to be attributable to the hypoglycæmia produced by insulin. The fact that small injections of dextrose suppress them supports this view.

Experiment 24.—Cat, 3·48 kgm. Eviscerated spinal preparation. The muscles of the right side were removed at the beginning of the experiment, and 10 units of insulin were then administered, two subsequent similar doses being given during the " insulin period." The muscles of the left side were removed 295 minutes later. The initial blood sugar was 0·310 per cent. After 105 minutes of insulin action the blood sugar was 0·086 per cent. After 175 minutes it was 0·052 per cent., and the sugar did not exceed this value during the rest of the experiment. The average glycogen content of the muscles removed at the beginning was 0·684 per cent., of those at the end 0·624 per cent.—a decrease of 0·06 per cent.

Experiment 25.—Cat, 4·135 kgm. Spinal preparation not eviscerated. The muscles of the right side were removed at the beginning of the experiment, and 10 units of insulin were then administered, 15 additional units being given 1½ hours later. The corresponding muscles of the left side were removed 5 hours and 30 minutes after the first dose of insulin. The initial blood sugar was 0·36 per cent., but it decreased rapidly, and after 3¼ hours of insulin action was 0·050 per cent. The sugar then remained below this level until the experiment was terminated 2¼ hours later. The average glycogen content of the muscles removed at the beginning was 0·448 per cent., of those at the end 0·424 per cent. —a decrease of only 0·024 per cent.

The figures for muscle glycogen show a decrease so small as to be of doubtful significance. Practically it may be said that the muscle glycogen is unchanged. The results of the earlier experiments make it probable that, in the preliminary stages of these, when sugar was disappearing rapidly under insulin, there may have been a corresponding small deposition of glycogen, and that this may have been followed by a secondary return to slightly below the original value, to meet the metabolic need of the tissues, raised by the occurrence of some convulsions. However that may be, it is clear that, under these conditions, doses of insulin greatly excessive in relation to the sugar available, and maintaining a severe hypoglycæmia for several hours, do not cause a significant depletion of the glycogen store, or a large conversion of carbohydrate into unidentified forms.

The Effect of Convulsions on the Glycogen Content of the Skeletal Muscles.

In many of the investigations from which the conclusion has been drawn that the glycogen of the skeletal muscles is depleted by the administration of insulin, the possible effect of the hypoglycæmic convulsions has not been eliminated. We therefore made a series of experiments, in which lethal doses of insulin were administered to rabbits in which one sciatic nerve had been cut, under ether anæsthesia, a few days previously.* The animals died after periods of varying length, in which violent convulsions alternated with intervals of, at first, partial recovery, and later coma. Immediately after death the tibialis and gastrocnemius muscles of the denervated and normal sides were removed and analysed for glycogen. The muscles of the normal side were removed first. The results are shown in Table XI.

To show that denervation of itself will not produce any corresponding contrast, one sciatic was cut under ether in each of 2 rabbits, which were kept for 3 days under the same conditions as the insulin rabbits, and then killed, No. 1 by a blow on the neck, No. 2 by coal gas. The glycogen analyses are shown in Table XII.

These results demonstrate very convincingly that insulin convulsions play a very important rôle in the depletion of the muscle glycogen. In the denervated muscles, which participated only passively in the convulsions, the glycogen content was within normal limits in spite of the prolonged hypoglycæmia.

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* These operations were performed for us by Dr. Dale.

Table XI.

Rabbit.	Amount of insulin.	Interval between injection of insulin and time of death.	Glycogen percentage.			
			Normal side.		Denervated side.	
			Tibialis.	Gastroc.	Tibialis.	Gastroc.
No 1	20 units	7 hrs.	<0·01	0·025	0·732	0·392
„ 2	20 „	11 „	0·161	0·274	0·445	0·457
„ 3	10 „	3 „	<0·01	0·180	0·755	0·640
„ 4	10 „	6½ „	<0·01	0·025	0·305	0·355
„ 5	10 „	8 „*	0·171	0·04	0·760	0·563

* Animal killed.

Table XII.—Control Rabbits.

Rabbit No.	Muscle.	Normal.	Denervated.
1	Gastroc.	0·477	0·473
2	Gastroc.	0·445	0·466
	Tib. Ant.	0·497	0·491
	Av.	0·473	0·476

Discussion.

The puzzle presented by the action of insulin has been of the following kind. In the diabetic animal insulin revives the missing function of storing carbohydrate in the form of glycogen; in the normal animal, on the other hand, most of the available evidence seemed to indicate that, so far from promoting such storage, insulin caused the disappearance from the body of glycogen already present. With the exceptions mentioned in the introduction, no observer had been able to detect storage of glycogen, even as a preliminary phase in the effect of a large dose of insulin on the normal animal. The prevalent view, accordingly, was that the carbohydrate, disappearing from the circulation and from the glycogen stores of the normal animal treated with excess of insulin, must be deposited in the body in some other form, in which it was temporarily withdrawn from oxidative metabolism. On that view it would be necessary to suppose, either that the deposition of glycogen in the diabetic under insulin is a relatively late result of the action, and does not represent the immediate effect of insulin on the carbohydrate metabolism, or else that the action of insulin in the diabetic organism, where the sugar available is large in proportion to the insulin, is qualitatively different from its action on the normal animal, where insulin is in excess in relation to the available sugar.

The first of these two possibilities our experiments may be said to have settled. The accelerated combustion of dextrose and synthesis of a further quantity to glycogen, seen in the diabetic subject, represent its immediate effects. The changes in blood phosphate suggest that the sugar becomes a phosphoric ester in a stage in the synthesis, but there is no significant storage in that form, and the sugar lost from circulation, and not oxidized, can apparently be found as glycogen in the muscles. The evidence for the oxidation of what is not stored as glycogen will be presented in a later paper. The demonstration of glycogen formation has been made possible by the nature of our experiments. The use of the spinal preparation has eliminated the complications due to convulsions, and when the preparation has also been eviscerated the problem has been reduced to its simplest terms, since the dextrose to be accounted for has been, in addition to that originally present in the body, the accurately measured amount infused. The observation that, while the muscles of different animals and even different muscles of the same animal vary widely in their glycogen load, that of corresponding muscles from the two sides of the same animal is always practically identical, gave us an accuracy of control which no method of observation can afford, in which one animal, or one set of animals, is used as control for another.

Lest it should be supposed that, by using eviscerated preparations, we were dealing only with the conditions presented by the diabetic animal, and that our results are therefore not applicable to the normal one, it should be noted that in spinal preparations not eviscerated we have obtained essentially the same results. The one point which our experiments do not make clear is the part played by the liver, under normal conditions, in the immediate storage of dextrose as glycogen in response to insulin. Our experiments, at their face value, would seem to indicate that the liver takes little part in this primary effect; indeed, in the one case in which an attempt was made to concentrate the action on the liver, by allowing the dextrose and insulin to pass through this organ on their way to the general circulation, the net result was a loss of a large amount of glycogen from the liver, and its deposition as glycogen in the muscles. We think it may fairly be argued, however, that the conditions of our experiment are unphysiological, in exposing the liver to persistent splanchnic stimulation, and that the results are no more indicative of its true function than the many failures to demonstrate storage of glycogen under insulin action in livers artificially perfused. It is still an open possibility that the storage of glycogen in the liver of the diabetic animal under insulin is a later and indirect effect; but our experiments cannot be held to prove the point. The one point which they appear definitely to establish is the immediate storage in the muscles, as glycogen, of sugar removed from the circulation by the action of insulin, either in the eviscerated preparation, or in that which is still normal to the extent that the pancreas and liver are intact. We agree with Lesser and his co-workers, therefore, whose results on whole mice have led them also to the conclusion that the removal of circulating dextrose by insulin in the normal, as in the diabetic organism, is produced by the oxidation of some and the synthesis to glycogen of the remainder.

In order to obtain a clearly recognizable increase of glycogen in the muscles, it was necessary to infuse abundance of dextrose to meet the insulin, and thereby to prevent any serious depression of the blood sugar below the normal level. But our experiments were not all of this kind. Even when the blood sugar was allowed to fall, under insulin, to a very low level, and to remain there for a long period, the glycogen of the muscles was not perceptibly depleted. When the sugar available was insufficient to produce a definite increase, the glycogen remained practically unaltered. Under the conditions of our experiments, therefore, with the animal immobilised by destruction of the brain, insulin even in large excess does not cause glycogen to be removed from the muscles and stored in another form. It must be supposed, therefore, that the disappearance in the normal animal is not due to a direct action of insulin, but to a call on the carbohydrate reserves by the metabolic requirements of the normally active organism, greatly intensified, as we have shown, by convulsions when they occur. The full discussion, however, of the relation of the effects here recorded to those observed in the normal, intact animal, may be deferred to a later paper, for which more complete data will be available.

Summary and Conclusions.

1. The disappearance under insulin of inorganic phosphate from the blood of the eviscerated spinal preparation is related to the disappearance of sugar, and not to the fall in sugar concentration in the blood.

2. No significant portion of the sugar which disappears from the blood of this preparation, under the influence of insulin, is stored in the muscles as a phosphoric ester.

3. When an abundance of sugar is available, a large proportion of the sugar which disappears from the blood of the spinal cat is deposited as glycogen in the skeletal muscles of the animal.

4. Insulin hypoglycæmia, lasting for from one to three hours, does not appreciably lower the glycogen content of the resting skeletal muscles of the spinal cat.

5. The decrease or complete disappearance of the muscle glycogen of normal rabbits, produced by large doses of insulin, is chiefly due to the convulsions.

It is a pleasure to acknowledge the active direction and kindly criticism of Dr. H. H. Dale throughout this investigation.

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Oxidation and Storage of Glucose under the Action of Insulin.

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Experiments published by three of us (B., H., and M.) (1) have demonstrated that, in a spinal animal with the muscles at rest, a large part of the glucose disappearing from the circulation under the action of insulin is deposited as glycogen in the muscles. The view that the remainder, which was in most cases the larger, and in some cases the much larger part, had been oxidized, was quite compatible with the observations of Burn and Dale (2), who had measured the oxygen consumption, but not the glycogen increase, under similar conditions. It was important, however, to put this possibility to a quantitative test by simultaneous measurement of the consumption of oxygen and the accumulation of glycogen in the same preparation. The experiments here described represent such an attempt to make a complete experimental balance sheet, representing the fate of the whole of the glucose.

Methods.

The methods of making the preparation, infusing glucose, and taking samples for analysis were identical with those described in earlier papers ((1) and (2)), the eviscerated spinal cat being used in all cases.

For measurement of the oxygen consumption we had the advantage of using a circulating respirometer, recently designed and constructed for the purpose by our colleague, Dr. E. H. J. Schuster. In principle it is quite similar to that which he previously designed (3), and which was used by Burn and Dale, but the details of construction have been so altered that the whole apparatus can be easily accommodated at the end of the operating table. The double-barrelled piston-pump of the earlier design is replaced by a two-chambered diaphragm-pump, with mechanically operated and synchronized valves. The pistons of both chambers are operated by a common crank-shaft, and the throw can be altered, if necessary, without stopping the pump, by turning a micrometer screw, which alters the position of the fulcrum. The wedge-shaped gasometer, previously used, is replaced by a counterpoised, cylindrical gasometer, into which, as before, the expired air is driven through a tower filled with soda-lime. When the gasometer, through absorption of oxygen, and consequent reduction of volume of the whole system, has fallen to a certain level, the counterpoise lifts a light trigger, bringing into play a clutch, and producing one stroke of a small accessory pump. This delivers a definite volume (4·016 c.c.) of oxygen into the gasometer, and the resulting fall of the counterpoise throws the clutch out of action, until shrinkage to the same volume again brings it into play. The strokes of the accessory oxygen-pump are registered by a mechanical counter, and the number occurring in each five-minute period was recorded in the notes. At the end of the experiment the total volume in cubic centimetres introduced in any period, representing the volume of oxygen absorbed in that period, could be calculated by multiplying the total number of strokes by 4·016, and then reducing to 0° and 760 mm.

In most experiments two doses, each 10 units, of insulin were injected by the saphena vein, the second being given 1½ hours after the first. In the later experiments, for reasons which will become clear, we found it necessary to determine the free glucose in the muscles, before and after insulin, as well as the glycogen. It was decided also to analyse samples from the liver, though the afferent vessels to it had been tied, in order to control the slow leakage of glucose from this source into the circulation during the experiment. For the determination of muscle-sugar some of the samples of muscle taken were divided into two, one being dropped immediately into hot potassium-hydrate solution, for determination of the glycogen, and the other, weighing about 5 grams, into 20 c.c. of cold alcohol in a centrifuge-tube, in which the alcohol had been cooled below 0° C. in a freezing mixture. The weight of the sample of muscle was determined by weighing the tube of cold alcohol before and after its addition. The muscle was minced finely, under the alcohol, with scissors, these being finally washed into the tube with a further 20 c.c. of cold 70 per cent. alcohol. The mixture was allowed to stand for about one hour, and then thoroughly centrifuged. The total volume was taken to be 40 c.c. + the number of c.c. corresponding to the weight of the muscle sample, reckoned as water. It was assumed that the free sugar was distributed equally between the solid and liquid constituents of this total volume. The clear alcoholic extract was decanted off from the compacted solid layer, and 35 c.c. were taken. This was evaporated to dryness on the water bath and taken up in about 15 c.c. of distilled water. 5 c.c. of 0·5 per cent. dialysed iron was then added, in order to remove proteins and other colloidal constituents, and the mixture was diluted to 25 c.c. and filtered. In the water-clear filtrate, suitably diluted, glucose was determined by the Hagedorn-Jensen ferricyanide method, which seems to be less sensitive to other reducing or inhibiting substances than any of the methods depending on reduction of cupric oxide. From the figure so obtained the "free sugar" per gram of muscle was calculated. It may be doubted whether the attribution of the whole of the observed reduction to glucose is strictly justifiable; but the object of the determinations being essentially comparative, and the glucose being the only muscle constituent likely to vary significantly under the conditions of the experiment, the difference between the values obtained from corresponding muscles, before and after insulin, probably represented the change in the content of free glucose with an accuracy comparable to that of comparative blood sugar determinations.

The liver samples were treated in the same way, except that, in this case, separate samples were not taken directly into hot potash for determining the glycogen, but the solid residue, after removal of the cold alcoholic extract, was dried to remove alcohol and then worked up by Pflüger's method for glycogen. The glycogen being determined as glucose, the figure obtained was added to that for the free glucose in the alcoholic extract, and the total carbohydrate remaining in the liver, when the sample was taken, was thus calculated. Again the difference between two successive such estimates gave a reasonable figure for the glucose escaping into the blood from the *cul-de-sac* of the liver vessels in the interim.

Results.

In the first two experiments the analyses made and the calculation of results from them followed the same lines as those adopted in the earlier paper. In the period before the injection of insulin we observed in each case, as Burn and Dale had observed in similar experiments, a consumption of oxygen much in excess of that required to account for the apparent use of glucose, as calculated from the quantity infused and the change in the blood sugar. The meaning of this discrepancy will become clear later, and there is no need to record the figures so obtained. A similar discrepancy, though much smaller in proportion to the total quantities concerned, appeared in the insulin period, when the calculated total glucose disappearing was compared with the sum of the glycogen accumulated in the muscles and the glucose equivalent of the oxygen used, as may be seen from the following experimental details.

Experiment 1.—Weight of cat = 2,450 g. Total period of insulin action, 3 hrs. 10 min. 6 per cent. glucose infused at an average rate of 3·6 c.c. in each 10 minutes; 68·4 c.c. in all = 4·104 glucose.

Blood sugar fell from 0·410 to 0·360 per cent. "Active volume" of body fluids calculated as 408 c.c., so that additional glucose loss
= 4·08 × 0·05 = 0·204 g.
Total calculated loss of glucose ... ... = 4·104 + 0·204 = 4·31 g.

Glycogen percentage in muscles—Before insulin = 0·804
Final = 1·102
Gain = 0·298

Weight of muscles = 1,225. Total gain of glycogen = 12·25 × 0·298 = 3·65 g.
Oxygen used, at 0° and 760°, = 1,969 c.c.
0·75 L. oxygen is equivalent to 1 g. glucose.
Glucose equivalent to oxygen used = 1·969 × 4/3 ... ... = 2·63 g.

Total glucose accounted for ... ... = 6·28 g.

Glucose accounted for, in excess of calculated loss ... ... = 1·97 g.

Experiment 2.—Weight of cat = 3,180 g. Total period of insulin action, 3 hrs. 49 min. 8 per cent. glucose infused at an average rate of 3·4 c.c. in each 10 minutes; 78·0 c.c. in all = 6·24 g. glucose.

Blood sugar fell from 0·390 to 0·260 per cent. = 0·130 per cent.
"Active volume" taken as 533 c.c., so that total additional loss of glucose
= 0·130 × 5·33 = 0·693 g.
Total calculated loss of glucose ... ... = 6·24 + 0·693 = 6·933 g.

Glycogen percentage in muscles—Before insulin = 0·842
Final = 1·154
Gain = 0·312

Weight of muscles = 1,590 g. Total gain of glycogen = 0·312 × 15·9 = 4·96 g.
Oxygen used, at 0° and 760° = 2,573 c.c.
Glucose equivalent to this = 2·573 × 4/3 ... ... = 3·43 g.

Total glucose accounted for ... ... = 8·39 g.

Glucose accounted for, in excess of calculated loss ... ... = 1·46 g.

The fact that the glucose accounted for was regularly and materially in excess of that which had been calculated as disappearing clearly indicated a defect in some part of the calculation. The "credit" side of the account was unlikely to be in excess. The glycogen estimate, though far from exact, could not be expected to be regularly too high. Pflüger's method is far more liable to errors of defect than of excess ; and while it is true that the increase observed in four separate muscles of the hind limb was assumed to represent the average increase over all the muscles of the body, it is also the case that the hind limbs of the spinal preparation are more prone than any other part of the body to small twitches and spasmodic movements, so that the accumulation of glycogen in their muscles would be expected to be less, rather than greater, than that in the rest of the body. In the measurement of the oxygen used there could be no serious error, except one due to a leak in the system, which was always carefully excluded. It was ascertained, by a direct test, that when, at the conclusion of an experiment, the circulation was stopped by incising the heart, the recorded absorption of oxygen ceased completely within a few minutes. Nor was there any good ground for supposing that substances other than glucose were being oxidized in significant amount. In a series of experiments made under the same conditions, Burn and Dale had found the respiratory quotient of the preparation to be exactly unity.

Since then there was no ground for supposing that the estimates of carbohydrate stored as glycogen and oxidized were too large, it was probable that the estimate of glucose which disappeared was too small. Here there were two fairly obvious sources of possible error.

(1) It had been assumed by Burn and Dale, and in calculating the results of all our own earlier experiments, that the liver, having its circulation arrested, could make no further contribution to the circulating carbohydrate. The liver, however, when its afferent vessels are tied, is not rendered immediately and completely bloodless. Stagnant blood remains in the portal capillaries and the efferent veins, and slowly drains into the vena cava. It seemed not unlikely, therefore, that the liver of the eviscerated preparation, its glycogen being rapidly converted to glucose through asphyxiation of its cells, would slowly, but continuously, contribute additional sugar to the blood of the vena cava by gravity and diffusion. We decided to test the possibility by estimating the carbohydrate in samples of the liver at different stages of the experiment, adding the calculated loss from the organ to our estimate of the glucose disappearing.

(2) Our calculations of the loss of glucose represented by a certain observed fall in the blood sugar percentage had been based on the purely empirical observation of Burn and Dale that, when a known amount of glucose was injected, without insulin, into the blood stream of the preparation made from a cat weighing 3 kilos., it produced such a rise in the blood sugar as would be

represented by its distribution over a volume of 500 c.c. This led to the assumption that any change observed in the blood sugar represented the loss from, or gain to, the system of such a total amount of glucose as would cause this change in a volume of 500 c.c. per 3 kilos. original body weight. There was no reason to suppose that this method of calculation would give more than a very rough approximation to the truth. Especially in dealing with changes produced by insulin, it was clearly desirable to substitute for it a direct determination of changes in the glucose content of the tissues, as well as in those of the blood. It was not possible to take samples of all the tissues in the body, but fortunately we were dealing with a preparation in which the skeletal muscles constituted by far the largest part of the tissues supplied by the circulation, and practically the whole of those having an active carbohydrate metabolism. We decided, therefore, on a new method of estimation, in which the blood sugar changes were taken to apply only to the calculated volume of the blood itself, and the free glucose in the muscles was determined separately, by the method indicated in an earlier section. The total loss of glucose entailed by an observed fall in the percentage of muscle sugar could then be calculated by use of the same factor as that employed in calculating the total gain of muscle glycogen.

Some preliminary experiments were made to determine the regularity of the distribution of free glucose among the muscles, and the degree to which it changed with changes in the blood sugar, whether produced by insulin or otherwise.

Experiment 3.—A spinal preparation was taken and eviscerated, and left to itself under artificial respiration on the warmed table for 5½ hours, without the supply of glucose from without or administration of insulin, so that the disappearance of glucose was only what was produced by the metabolic needs of the tissues of this preparation. The blood sugar fell from the initial level of 0·300 per cent. to 0·132 per cent. in 3½ hours, and to 0·068 per cent. in 5½ hours. Samples of four muscles were taken in the usual manner at the beginning, and of the corresponding four muscles at the end of the experiment. Their average glycogen content remained practically constant, that of the initial samples being 0·736 and that of the final samples 0·777 per cent.—a difference within the errors of sampling and analysis. The figures obtained for free glucose percentages in the muscles were as follows :—

	Initial.	Final.
Gastrocnemius	0·215	0·210
Tibialis ant.	0·225	0·242
Rectus femoris	0·227	0·237
Vastus internus	Lost.	$\boxed{0{\cdot}217}$
Average (omitting last)	0·222	0·229

It will be noted that the value is very constant over the different muscles, and that it remains practically unchanged during the period in which the blood sugar sinks from 0·3 to 0·068.

Experiment 4.—This experiment figures already in the paper by Best, Hoet and Marks as Experiment 23. It was there pointed out that the accumulation of glycogen, produced in the muscles by the action of insulin, was in this case more than sufficient to account for the glucose infused and that which disappeared from the blood, on the basis of calculation then in use. The full figures for glycogen and free sugar in the muscles analysed were as follows :—

	Before insulin.		Final.	
	Glycogen.	Free glucose.	Glycogen.	Free glucose.
	Per cent.	Per cent.	Per cent.	Per cent.
Gastroc.	1·090	0·236	1·127	0·173
Rect. fem.	0·953	0·332	1·059	0·191
Vast. int.	0·522	0·258	0·758	0·181
Tib. ant.	Lost	0·220	$\boxed{0{\cdot}900}$	0·181
Average	0·855	0·262	0·981	0·182

Average increase in glycogen = 0·126 per cent.
Average decrease in free glucose = 0·080 per cent.

It will be clear that two-thirds of the glycogen increase can be accounted for by the loss of free glucose from the muscles themselves; the absence, however, of data as to oxygen consumption and as to the contribution of extra glucose by the liver made it impossible to produce a full reckoning of the carbohydrate balance in this experiment. The values for free glucose in different muscles taken at the same stage are much more uniform than those for glycogen in the same muscles, the only really aberrant figure being that for one muscle (Rect. fem.) before insulin. Calculation of the total change from that in the sample can, therefore, be made with at least as much confidence for free glucose as for glycogen. Though the free muscle glucose in this experiment showed a substantial fall under insulin, it by no means kept pace with that of the blood sugar, which fell in the same period from 0·27 per cent. to 0·088 per cent. Nevertheless, the smaller fall in the percentage of muscle sugar makes a much larger contribution to the total of glucose lost than does the larger fall in the blood sugar percentage. The cat weighing 3·725 kilos., we may reckon that the blood volume was about 230 c.c., and that the muscles weighed about 1,800 grams.

Lost from blood .. .. $0{\cdot}182 \times 2{\cdot}3 = 0{\cdot}42$ g.
„ muscles .. $0{\cdot}08 \times 18 = 1{\cdot}44$ g.

Having thus a more satisfactory method for estimating the total amount of glucose disappearing, we proceeded to compare this total with that required by the observed use of oxygen and deposition of glycogen. We may consider first the light thrown, by the analyses of liver samples, on the discrepancy observed by Burn and Dale between the volume of oxygen absorbed by this preparation and the amount of glucose infused, to maintain the blood sugar at a constant level, without insulin. They found that oxygen was consumed in substantially greater amount than was needed to oxidize this glucose, though the respiratory quotient proved that nothing but carbohydrate was being oxidized. We have seen that, in the absence of insulin, the muscle sugar remains remarkably steady, so that the supply of extra glucose from the liver seems the only explanation for the discrepancy. The results of the first stage of Experiment 5 show that it is adequate.

Experiment 5.—Weight of cat = 3·2 kilos.

A. First stage—before insulin. The period of observation was 50 minutes. Samples of liver were taken for analyses at the beginning and end of the period. The blood sugar remained practically unchanged (0·24 per cent.), while 5·6 c.c. of 4 per cent. dextrose were given by slow infusion.

Weight of liver (less that of initial samples) = 70 g.

	Per cent.
Average of 1st samples—Glycogen 3·24 per cent. + Glucose 1·61 per cent.	= 4·85
Average of 2nd samples—Glycogen 1·26 per cent. + Glucose 2·35 per cent.	= 3·61
Loss of carbohydrate	1·24

Total loss of carbohydrate from liver ... ... = 1·24 × 0·7 = 0·868 g.
Glucose infused ... ... = 5·6 × 0·04 = 0·224 g.

Total glucose lost ... ... 1·092 g.

Oxygen absorbed—784 c.c. at N.T.P.
Glucose equivalent to oxygen used ... ... = 0·784 × 4/3 = 1·045 g.

It is obvious that the oxygen consumption shows as close a correspondence with the observed loss of glucose as could be expected with the methods of measurement available. The excess consumption of oxygen, observed by Burn and Dale, can be entirely accounted for by the contribution to the blood from the carbohydrates of the liver, which is nearly four times as great as the amount of glucose infused. The total loss of glucose, so calculated, appears, indeed, to be very slightly in excess of that oxidized. Even if the difference were a real one, however, the deposition of 47 mgm. as glycogen in some 1,600 g.

of muscle would be altogether beyond the possibility of detection by analysis.

B. Insulin period.—After the second liver samples and first muscle samples had been taken, 10 units of insulin were injected intravenously, and the rate of glucose infusion increased. Another 10 units were injected 1½ hours later, and the total period of insulin action was 2½ hours. The third liver and second muscle samples were then taken.

Weight of liver (less that of 1st and 2nd samples) = 58 g.

	Per cent.		Per cent.
Av. liver glycogen just before insulin	= 1·26.	Av. liver glycogen at end	= 0·70
„ free sugar „ „	= 2·35.	„ free sugar „	= 1·30
„ carbohydrate „	= 3·61.	„ carbohydrate „	= 2·00

Difference = 1·61 per cent.

Loss of carbohydrate from liver ... ... = 1·61 × 0·58 = 0·934 g.

Av. muscle glucose before insulin = 0·25

„ „ at end = 0·19

Loss ... ... = 0·06

Total loss of glucose from muscle ... ... = 0·06 × 16 = 0·960 g.

Blood sugar fall from 0·240 to 0·130 per cent. ... = 0·110 per cent.

Loss of glucose from blood ... ... = 0·110 × 2 = 0·220 g.

Total glucose infused ... ... = 3·250 g.

Calculated total loss of glucose ... ... **5·364** g.

Average muscle glycogen before insulin = 0·654 per cent.

„ „ at end = 0·830 „

Gain ... ... = 0·176 „

Total calculated gain of muscle glycogen ... ... = 0·176 × 16 = 2·82 g.

Oxygen used = 2,230 c.c. at N.T.P.

Glucose equivalent of oxygen used ... ... = 2·23 × 4/3 = 2·97 g.

Total glucose accounted for ... ... = **5·79** g.

On this calculation the glucose accounted for by glycogen formation and oxidation, 5·79 g., is slightly in excess of the detected loss, 5·364 g. The difference cannot, however, be regarded as having any true significance. The calculations of the total loss of muscle sugar and gain of glycogen, in particular, have not a high degree of accuracy. They depend on the multiplication of the figures obtained from sample analyses by the large factor 16. If we divide

0·426, the difference between the totals for glucose lost and accounted for, by 16, the result is 0·028 per cent., so that an underestimate of the muscle sugar loss by 0·014 per cent., and an overestimate of the glycogen gain by the same percentage, would account for the whole difference in the final totals. The comparison of one set of muscles with the corresponding ones of the opposite leg, either for free sugar or glycogen, is liable to inaccuracy at least as great as this. We cannot, therefore, regard the difference between the totals on the two sides of the final account as a real one; but we are entitled to conclude that *the glucose disappearing under the action of insulin is, in this experiment, fully accounted for by the sum of what is oxidized and what is deposited as glycogen.*

Experiment 6.—Weight of cat = 2,600 g. In this experiment no sample of the liver was taken until just before insulin was given, so that no balance can be made between glucose lost and oxidized in the pre-insulin period. As in all the other experiments, the oxygen absorbed in this period was much in excess of that required to oxidize the glucose infused.

Insulin period.—Four muscles and samples of liver were taken just before the first injection of 10 units of insulin, a second similar injection was given about 1 hour later, and a third after a further 1½ hours. The final samples were taken 3½ hours after the first insulin injection, the preparation being, therefore, under the action of insulin for this total period, and receiving 30 units in all. The infusion of glucose was in this case so greatly accelerated after insulin was given that the blood sugar did not fall, but rose somewhat, being over 0·44 per cent. during the whole period.

Weight of liver (less that of first samples) = 70 g.

	Per cent.		Per cent.
Av. liver glycogen just before insulin =	0·81	Av. liver glycogen at end =	0·71
„ free glucose „ „ =	1·4	„ free glucose „ =	1·04
	2·21		1·75

Difference = 0·46 per cent.

Total loss of carbohydrate from liver ... ... = 0·46 × 0·7 = 0·312 g.

Av. muscle glucose before insulin = 0·22
„ „ after „ = 0·20
Loss ... ... = 0·02

Total calculated loss of glucose from muscle ... ... = 0·02 × 13 = 0·260 g.
Glucose infused ... ... = 4·80 g.
5·372 g.

Blood sugar rose from 0·44 to 0·51 = 0·07 per cent.

Total *gain* of glucose in blood ... ... $= \frac{0\cdot07 \times 167}{100} =$ 0·117 g.

Calculated total loss of glucose ... ... = 5·255 g.

Per cent. Per cent.

Av. muscle glycogen before insulin = 0·661 per cent.
„ „ at end = 0·851 „

Gain = 0·190

Total calculated gain of muscle glycogen ... ... = 0·190 × 13 = 2·470 g.
Oxygen used = 1,946 c.c. at N.T.P. = 1·946 × 4/3 g. glucose ... = 2·595 g.

Total glucose accounted for ... ... = 5·065 g.

In this case the apparent difference between the two sides of the account is in the other direction, since we account for 190 mgm. less glucose than the estimated loss; but again the difference has no significance in relation to the accuracy of the method of calculation. The recorded fall in free muscle sugar —0·22 to 0·20 per cent.—cannot be regarded as a certain difference; yet it contributes 0·260 g. to the balance sheet as presented, and its absence would change the apparent discrepancy to the other side of the account.

In the two complete experiments thus far recorded, the infusion of glucose was raised to such a rate, after insulin was first injected, that the blood sugar percentage, falling in Experiment 5 and rising in Experiment 6, remained in both well above the normal level throughout the period of observation. It was important to know whether the balance would still hold good when the supply of glucose was so small in relation to the insulin given that the blood sugar would fall rapidly to a minimal value. These conditions were realized in Experiment 7.

Experiment 7.—Weight of cat = 2,800 g. Glucose was infused throughout the experiment at the rate of 40 to 48 mgm. in each 10-minute period, except for an hour in the middle of the insulin period, when the rate of infusion was gradually raised, in order to test its effect on the rapid decline in the rate of oxygen absorption, which had appeared about 40 minutes after the first insulin injection. The effect on oxygen usage being small, the rate of glucose infusion was dropped again, and continued at the original slow rate for a further 2½ hours. 25 units of insulin in all were administered, 10 units at the first injection, 5 units 40 minutes later, when the rate of glucose infusion was increased, and another 10 units 1½ hours after the second injection. The blood sugar percentage fell from 0·360 per cent. at the time of the first dose of insulin to 0·034 per cent. at the end of the experiment, 4 hours 10 minutes later. The first samples of liver and four muscles of one leg were removed shortly before insulin was first injected, and the rest of the liver and the four corresponding muscles at the end of the experiment.

Weight of liver (less that of first sample) = 102·6 g.

	Per cent.		Per cent.
Av. liver glycogen just before insulin	= 1·285	Av. liver glycogen at end	= 0·625
,, free glucose ,, ,,	= 1·760	,, free glucose ,,	= 1·570
	3·045		2·195

Difference = 0·850 per cent.

Total loss of carbohydrate from liver ... ... = 1·026 × 0·850 = 0·872 g.

Av. muscle glucose before insulin = 0·320 per cent.
,, ,, at end = 0·240 ,,

Loss = 0·080 ,,

Total calculated loss of free glucose from muscle = 0·080 × 14 = 1·120 g.
Total glucose infused ... ... = 1·680 g.
Blood sugar fell from 0·360 to 0·034 = 0·326 per cent.
Total loss of glucose from blood = 0·326 × 1·75 ... ... = 0·570 g.

Calculated total loss of glucose ... ... = 4·242 g.

Av. muscle glycogen before insulin = 0·727 per cent.
,, ,, at end = 0·799 ,,

Gain = 0·072 ,,

Total calculated gain of muscle glycogen ... ... = 0·072 × 14 = 1·008 g.
Oxygen used = 2,309 c.c. at N.T.P. = 2·309 × 4/3 g. glucose ... = 3·079 g.

Calculated total glucose accounted for ... ... = 4·087 g.

Again the two totals, of glucose lost and of glucose found as glycogen or oxidized, show as near an identity as it is reasonable to expect with the method, so that we can safely draw the conclusion that *even when insulin is present in such excess, in relation to the supply of glucose, that a severe hypoglycæmia is produced, all the glucose which disappears is either oxidized or stored as glycogen, no significant part of it being deposited in an unrecognized form.*

It is, of course, possible, and even probable, that a very small part of the disappearing glucose forms an addition to the floating balance of hexose phosphate, on its way to storage as glycogen. Our experiments show definitely, however, that no significant proportion is held at any one moment in this form. A point deserving mention is the curious lack of parallelism between the percentages of blood sugar, on the one hand, and "free glucose" in the muscles on the other, seen in every experiment in which the latter was determined. It would clearly be unprofitable to discuss the meaning of the disparity, without fuller evidence as to whether other substances, and if so what substances and in what proportions, contribute to the reduction on which this figure is based. The point is one worth further investigation. For our present purpose it seemed quite justifiable to assume that a fall in the value is due to loss of glucose, just as it is assumed that a fall in the reducing power of a blood-filtrate is due to loss of glucose, though it is known that a small proportion of other reducing substances is present.

Discussion.

The results of the experiments are so definite that they need no discussion of themselves. Under the conditions chosen, with a preparation consisting essentially of naturally perfused quiescent skeletal muscle, all the glucose disappearing under insulin is either oxidized or stored in the muscles as glycogen.

The important question remains, Does this action of insulin, thus artificially isolated in our experiments, cover the whole of its effects as seen in the normal animal ? It is quite certain that it does not. Failure to detect glycogen storage could easily be explained ; but the fact calling for explanation is the very obvious decrease, often the complete disappearance, of glycogen from the normal animal receiving an excessive dose of insulin. That the convulsions consequent on hypoglycæmia greatly accelerate this depletion of the muscle glycogen, and thus help to annul the effect of any initial storage action, has been clearly demonstrated in a former paper (Best, Hoet and Marks (1)). But the fundamental difficulty remains, namely, that, if this exhaustion of the carbohydrates is attributed to oxidation, additional to that of the normal resting metabolism, the total respiratory exchange must increase, and the body temperature would be expected to rise. In fact this exhaustion is accompanied by a fall in the respiratory exchange, even to as little as one-third of its normal resting value, and by a corresponding fall in the body temperature. These effects can be well seen in the experiments recorded by Dudley with Laidlaw, Trevan and Boock (11), and with Marrian (12), and also in those experiments of Lesser (10), in which he used doses of insulin sufficient to produce " symptoms."

There are two possible explanations for this paradox of accelerated loss of recoverable carbohydrate with concurrent depression of oxidative metabolism. The one which has apparently been found acceptable by most workers on the subject is the supposition that, when insulin is present in excess, the synthesis of dextrose to glycogen is not completed, but stopped at some intermediate stage, or diverted along some other path, so that the carbohydrate is deposited in some unidentified form, in which it is withdrawn from oxidative metabolism, and is either destroyed or not extracted by the methods ordinarily used in analysis. This is the conception provisionally adopted by Macleod, in his exhaustive reviews of the subject ((4) (5)). It must be admitted that such temporary immobilisation of the carbohydrate, if there were direct evidence of its occurrence, would account for some of the effects observed. The supposition, however, is directly against the evidence of the experiments here presented. Under conditions which should have been ideal for its detection, since relatively large quantities of glucose were made to disappear under the action of insulin, we observed no trace of this conversion into unrecognized forms; all that was not oxidized was recovered as glycogen. The supposition further involves the difficult assumption that insulin, when present in excess, in relation to the carbohydrate available, has an action *qualitatively* different from that which it produces in the presence of abundant carbohydrate.

The second possible explanation seems to us to have a much clearer relation to the known facts. It is suggested by a consideration of the abnormalities of metabolism which the organism presents when insulin is defective or absent—*i.e.*, in natural or artificial diabetes. Under such conditions the body presents two functional defects—defective use and storage of carbohydrate on the one hand, and excessive, wasteful formation of carbohydrate from proteins (and possibly also from fats) on the other. We have no certain knowledge as to the relation of these two effects to one another, such as would entitle us to regard one as a secondary consequence of the other. But we do know that both these defects are removed by insulin in appropriate dosage; and it has been shown to act in both these directions not only in the diabetic patient and in the depancreatized animal, but also in the dog poisoned by phloridzin (Ringer (6), Nash (7)). It is reasonable to suppose that insulin in excessive doses will produce both these, its normal effects, to an abnormal degree.

(1) We should expect it to accelerate the combustion of carbohydrate, and particularly the associated storage as glycogen, to a rate much above the normal. That it has this effect we believe that our experiments, in addition to those of Lesser and his co-workers ((8) (9)), have sufficiently demonstrated.

(2) We should expect it to depress the new formation of carbohydrate from other substances in the liver to a rate much below the normal. We believe that this effect is clearly evident in a large number of published experimental results, which have shown a depression of total respiratory metabolism, associated with a large rise in the respiratory quotient. The clearest examples, perhaps, are those given by Laufberger (13) (14). So much was he impressed by their significance that he made at first the suggestion that this stoppage or restriction of the new formation of

carbohydrate was the sole effect of insulin. This suggestion obviously would not fit the facts any better than the suggestion that the sole effect is to promote combustion and synthesis.

It is probably not realized by those who have not examined the figures how small a ration of total carbohydrate, in relation to the metabolic need, is present at any one moment in the body of such an animal as a fasting mouse. Bissinger and Lesser's analyses show that it amounts to about 170 mgm. per 100 grams of body weight. On the other hand, if the mouse were using carbohydrate alone for its metabolism, it would require about 560 mgm. per 100 grams per hour to maintain its normal rate of expenditure. It is obvious, then, that the normal metabolism can only be maintained by the rapid new formation of carbohydrate in the liver. If this new supply is stopped, the existing supply will be exhausted in about 20 minutes, unless the rate of metabolism is rapidly reduced. The effects which follow the injection into such a mouse of an excessive dose of insulin are exactly what, under such conditions, we should expect. There is an initial sharp rise of the respiratory quotient, indicating that, owing to depression of new formation, the oxidative metabolism has been concentrated on the pre-existing carbohydrate. Other carbohydrate is probably, during a very brief period, being removed from circulation to be deposited as glycogen. But the needs even of the rapidly falling metabolism soon begin to call on all the carbohydrate reserves, and the onset of hypoglycæmic convulsions increases the rate of their exhaustion. If the dose has been large enough, and the fall of metabolism is delayed by putting the animal into a thermostat at 37°, it may be expected that the mouse will die in about half an hour, with its initial stores of carbohydrate completely exhausted; and this is what, in fact, occurs.

In the fasting normal mouse or rat, with their rapid metabolism, it is obviously the depression of new carbohydrate formation which will form the most obvious feature in the action of an excess of insulin, and the only one, indeed, which experiment may succeed in detecting. In the diabetic subject both the effects are equally in evidence. In the eviscerated spinal preparation, as used in our experiments, we have artificially eliminated, from the outset, the new formation of carbohydrate, and the preparation is carrying out its metabolism wholly at the expense of carbohydrate originally present in the body as such, or supplied from outside by infusion, so that the oxidation and synthesis are seen isolated from the other effect, which so often complicates their demonstration in the normal animal.

Our experiments can give no indication of the extent to which the liver participates in the storage of glycogen under insulin. v. Issekutz (15), alone among those who have made such experiments, appears to have obtained evidence, on the perfused frog's liver, of a direct inhibition by insulin of the conversion of glycogen to glucose. This is in accord with the demonstrated reappearance of glycogen in the liver of the diabetic animal treated with insulin. Insulin has also been shown to cause storage of glycogen in the liver of an animal under phloridzin (Cori (16)), and, when given in small doses, of a normal fasting animal (Frank, Hartmann and Nothmann (17)). The fact, which we and others have observed, that rabbits dying of insulin convulsions often have a substantial remainder of glycogen in the liver, though that of the muscles is exhausted, may possibly point in the same direction. Since the liver-glycogen, however, is the primary carbohydrate reserve of the body, there must always be a conflict, when insulin is present in excess, between a direct promotion of storage by insulin in that organ, and the promotion of glycogenolysis by the hypoglycæmia, acting either directly or through the suprarenal glands. When to this conflict there is added the effect of depressed new formation of carbohydrate, it is not surprising to find that the end-result of the action of insulin on smaller animals, such as mice, with their rapid metabolism, is often a complete exhaustion of the glycogen from the liver as well as from other organs.

On these lines, attributing to insulin when present in excess no other action than the production of its known physiological effects with more than physiological intensity, and without any assumption of unknown and unrecognizable forms of carbohydrate, we believe that it will prove possible to account for all its effects, in any dosage, and on any species. This view, in essence, was put forward some years ago by one of us (H. H. D. (18)) and in greater detail by Lesser (19). Each in turn (Lesser (10), Dale (20)) later expressed doubt of its adequacy. In the light of more recent evidence Lesser (9) has returned to it, and we regard the evidence here presented as definitely confirming it.

Summary.

1. The glucose which disappears from an eviscerated spinal preparation under the action of insulin is equal to the sum of the glycogen deposited in the muscles and the glucose-equivalent of the oxygen absorbed.

2. This balance is preserved, whether the blood sugar is maintained at a high level by rapid infusion of glucose, or allowed to sink to a very low level by restricting the supply.

3. The view is advocated that the effects of insulin in excess represent an intensification of its physiological effects.

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Observations on the Onset of Rigor Mortis.

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These observations on rigor mortis were made from time to time on cats and rabbits dying after large doses of insulin, on rabbits dying as a result of prolonged thyroid feeding, and on spinal cats to which large doses of insulin had been administered to study the carbohydrate metabolism of the muscles.

Rabbits which have received large doses of insulin, and which, as a result, have had periods of convulsions and collapse, may either die very quickly or may survive for long periods. The exact cause of the death is still not clear. In most cases rigor mortis sets in immediately after death, and the onset is invariably much earlier than in a normal rabbit killed by a blow on the head. The same rapid onset of rigor mortis after death from insulin has, no doubt, been noticed by other workers in rabbits and mice, and† was mentioned by Baur, Kuhn and Wacker (1) as occurring in guinea-pigs. The death is always accompanied by marked hypoglycæmia.

Rabbits dying from prolonged thyroid feeding show a pronounced hypoglycæmia, the reduction showing a blood sugar of 0·03 per cent.—*i.e.*, practically none. As Burn and Marks (2) have noted, rigor mortis sets in immediately after death in such animals, as it does in animals dying in hypoglycæmic convulsions produced by insulin. The reasons of this rapid onset of rigor were not easy to find, in the light of current conceptions of the cause of this phenomenon.

Since the researches of Fletcher and Hopkins, lactic acid has been looked upon as the most obvious determining cause of muscular fatigue and of the stiffening of rigor mortis (3). On the basis of this conception, a plausible explanation for the rapid onset of rigor mortis in many conditions has been available. For example, in the case of the hunted fox or hare, or in a fatigued isolated muscle, or in a rabbit dying from strychnine convulsions, it has generally been supposed that the previous accumulation of lactic acid has accelerated the onset of rigor after death of the animal. Red muscles go into rigor later

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† So far as we are aware, the phenomenon was first publicly mentioned by Dr. J. W. Trevan, at a meeting of the Physiological Society.

than white muscles, but they shorten more. Fletcher has shown that the lactic acid is formed more slowly in the red muscle and is less abundant than in the white. The conclusion from this parallelism has been, that the speed of lactic acid formation and the quantity accumulating determine the speed of the onset of rigor, but not necessarily the intensity of the shortening (*v.* Fürth(4)) It is difficult to find an explanation of rigor after death produced by insulin, or by thyroid feeding, on the basis of this conception. Baur and Kuhn (5) found that the preformed lactic acid was reduced in the muscles of animals killed with large doses of insulin, while the glycogen, which is the ultimate source of the acid, was reduced practically to *nil*, as Dudley and Marrian (6) had previously observed.

With regard to the lactic acid in the muscles of animals dying after thyroid feeding, no data have hitherto been available. These animals die with practically no struggling and have very little available carbohydrate, so that a large accumulation of lactic acid in their muscles could not be expected.

The methods used in this investigation were identical with those used by Best, Hoet and Marks (7). For lactic acid Clausen's method was used.

Nature of the rigor.

We have not made a detailed study of the changes in the muscle which constitute the precipitate rigor with which we are concerned. In an ordinary rigor mortis the muscles undergo a progressive shortening and hardening, the shortening being particularly well seen when the antagonistic muscles are cut before the rigor is complete. In the case of the rigor which is here in question we have never observed this shortening on cutting the antagonists; but this is probably to be explained in part by the abnormally rapid completion of the effect. In many cases the animal becomes completely rigid at the moment of death, *i.e.*, as the circulation stops. The time of onset of the rigor has been taken as the time of appearance of a pronounced stiffening and hardening of the muscles. As to the time from onset to completion, it can only be said that it must be extremely short, and would need special methods for its detection and measurement. We have made no actual measurements of the tension developed by the muscles in this rigor, but simple observation gave the impression that it was much less than that seen in the rigor of the muscles of normal animals.

The rigor we are considering not only comes on, but also passes off with abnormal rapidity. This was also observed by Baur and Kuhn in the case of rigor following death from excessive doses of insulin. It may frequently be observed that the muscles have become soft and extensible again after about

2 hours, whereas the normal rigor, appearing some hours after death, often persists for as long as 36 hours. It must not be supposed, however, that this early flaccidity indicates that the condition is not a true rigor mortis. The muscle is dead, just as is the normal muscle becoming flaccid after its prolonged rigor. The whole series of changes, including those preceding the rigor, those occurring during its persistence, and those determining its final disappearance, appears to be very greatly accelerated.

Another feature of this rapid, evanescent rigor is that, if forcibly broken, it does not return.

Accelerated rigor following insulin.

When this phenomenon was first observed it was natural to connect it either with the presence of insulin, or with the profound hypoglycæmia produced by that hormone. It is easy to demonstrate, however, that neither of these is the directly determinant factor. In the experiment which we published with C. H. Best (7), we were concerned with the effects of insulin in producing disappearance of glucose from the blood and appearance of glycogen in the muscles of the spinal preparation, receiving large injections of insulin, with or without glucose. In some of these experiments the spinal cat was living for 2 to 4 hours under the action of excessive doses of insulin, with a blood sugar of 0·04 to 0·05 per cent. Yet when the experiment was terminated, and the circulation stopped by incising the heart, the preparation remained at room temperature for 2 hours without any sign of rigor mortis, and was not observed longer. It is clear, therefore, that neither insulin in the circulation nor the hypoglycæmia which it causes has any accelerating effect on the process producing rigor.

The muscles of the spinal preparation, which have not been affected by convulsions, differ from those of a normal animal dying after an excessive dose of insulin, in the fact that their glycogen content is normal, whereas that of the animal dying with convulsions is greatly reduced, and may be completely exhausted. It soon became clear that there was a direct relation between the exhaustion of the glycogen and the rapidity of the rigor. This was observed particularly well in the experiments, already published with Best, on the effects of insulin on the muscle glycogen of rabbits in which one sciatic nerve had been cut. The paralysed muscles received the same blood, rich in insulin and poor in glucose, as the corresponding muscles of the opposite leg; not having shared in the convulsions, however, they retained their glycogen, of which the normal muscles were to varying degrees depleted. The denervated muscles remained, in every case, perfectly flaccid and reactive for two hours at room temperature after death of the animal. The normal muscles, on the other hand, went into rigor mortis within 5 minutes of the death of the animal in 6 out of 9 cases. On analysis the muscles were found to have lost practically all their glycogen. In 3 other cases the normal muscles did not show this rigor within 5 minutes of death. They were found to contain a moderate amount of glycogen, though less than their denervated fellows. In these experiments the onset of rigor was timed in the muscles of the fore-limbs, and was found to be accelerated, though not to the extreme degree associated with exhaustion of the glycogen. The results of these observations are collected in Table I.

Table I.

Rabbit.	Interval between injection of insulin and time of death (in hours).	Glycogen percentage (normal side).		Onset of rigor.
		Tibialis.	Gastrocn.	
1	7	<0·01	0·025	< 5 min.
2—	3	<0·01	0·180*	,,
3—	6½	<0·01	0·025	,,
4	7	<0·01	—	,,
5	5½	<0·01	—	,,
6	5¼	<0·01	—	,,
7—	11	0·161	0·274	±30 min.
8—	8	0·171	0·04	±30 ,,
9—	7	0·368 (fore-limb)	—	>60 ,,

* Note that the gastrocnemius was not the muscle in which onset of rigor was timed.

R. 7— has evidently less convulsions than rabbits 1 to 6.
R. 9— had practically no convulsions.

The disappearance of glycogen was not accompanied by a corresponding accumulation of lactic acid. On the contrary, the lactic acid contents of the muscles showing the rapid rigor were low, values as low as 50 mgm. per cent. being found in muscles already in rigor (*see* Table VI). On this point our observations confirmed those of Kuhn and Baur (5).

The lactacidogen content was estimated in the muscles showing early rigor and in the corresponding denervated muscles of the other leg. The muscles were taken as quickly as possible after death, and samples were dropped at once into ice-cold 4 per cent. trichloracetic acid solution, for estimation of the free phosphoric acid, while other samples were minced in sodium bicarbonate solution, incubated in this for 18 hours, and then precipitated with trichloracetic acid, for estimation of the total phosphoric acid, the difference between the two values giving a measure of the lactacidogen present. Table II shows a set of such determinations, made on the denervated and the corresponding normal muscles from a rabbit dying of insulin convulsions.

Table II.

The figures represent inorganic phosphorus in mgm. per 100 gm. of muscle.

	Normal.		Denervated.
Gastrocnemius	A.—free	139	107
	B.—after digestion	141	142
	Lactacidogen	2	35
Tibialis ant.	A.—free	141	113
	B.—after digestion	156	156
		15	43

For these determinations the muscles of the normal side were removed before the denervated muscles. Nevertheless, the lactacidogen content of these normal muscles, which have shared in the convulsions and exhibited the rapid rigor, is very small, while that of the denervated muscles, though low, is within normal limits for muscles taken 10 minutes after death. The fact that the free phosphorus content of these denervated muscles is unusually high may reasonably be attributed to the poor circulation which they have received for some time before the death of the animal. It will also be obvious that the *total* phosphorus values for the corresponding normal and denervated muscles are identical.

Effects following prolonged feeding with thyroid gland.

Burn and Marks (2) have shown that, after daily administration for about three weeks of large doses of thyroid gland substance with the food (1·8 g. dry gland substance *per diem*), rabbits die spontaneously with a severe hypoglycæmia. We found that the muscles of such rabbits are completely free from glycogen, even the heart muscle showing the depletion. Fukuï (8) has recently examined the effect of feeding with thyroid gland on the muscle glycogen of rats, and has stated that the muscles do not lose their glycogen like the liver under this treatment. His experiments, however, were not carried to the stage at which the final exhaustion of the glycogen depôts, of the muscles also, occurs. Marks (9) has shown that when this advanced stage is reached, a very small dose of insulin, or the sudden introduction of a small dose of glucose into the blood, probably by provoking a sudden excessive secretion of insulin, will produce a rapidly fatal hypoglycæmia. It will be convenient to mention certain other changes, incidentally observed to accompany the terminal hypoglycæmia in these thyroid-fed rabbits, before dealing with the rigor mortis which follows it.

Body temperature.—When a fatal hypoglycæmia has been evoked in such animals by a small injection of insulin or glucose, a rapid rise in the body temperature can usually be observed during the last hour of life, and continuing after death. The temperature may rise from 39·5° C. (103° F.) an hour before death to reach 42·8° C. (109° F.) or even 43·3° C. (110° F.) after death. The effect has no connection with the rapid rigor mortis. Rabbits which die spontaneously, after feeding with thyroid to complete exhaustion of the glycogen reserves, do not exhibit it, and frequently have a subnormal temperature, though their rigor is equally rapid; on the other hand, rabbits killed at an earlier stage of thyroid feeding showed a post-mortem rise of temperature, though rigor did not appear in them with such extreme rapidity. The condition may probably be related to the abnormally rapid combustion of the carbohydrate remaining in the thyroid-fed animal. When complete exhaustion has been reached this can no longer occur.

The terminal hyperpyrexia has been observed in fatal cases of Graves's disease in man (10, 11). Dr. Cornelis, of Louvain, informs us that he has seen immediate onset of rigor mortis in a case of Graves's disease in which death occurred during operation.

Inorganic phosphate in the blood.—When glucose is administered to a normal animal a small consequent fall, by about 1 mgm. or so per 100 c.c., may be detected in the inorganic phosphorus of the blood. Insulin causes a larger fall, accompanying the disappearance of blood sugar. In thyroid-fed animals at an early stage of the treatment, when, as Burn and Marks have shown, the sensitiveness to insulin is diminished, no departure from these normal changes in the blood phosphate was observed. In the final stages, however, when the exhaustion of the carbohydrate reserves is approaching or has occurred, a rapid rise in the inorganic phosphorus of the blood is observed to accompany the terminal hypoglycæmia, whatever the determining cause of its onset. From a previous level of 6 to 9 mgm. per 100 c.c. the inorganic phosphorus rose in such cases to 10·8, 12, 15 and, in one instance, to 20 mgm. per 100 c.c.

Lactacidogen in muscle.—As in the case of rabbits dying after large doses of insulin, the muscles of rabbits dying of hypoglycæmia in the late stages of thyroid feeding have nearly all their phosphate in the free state. The additional phosphate liberated by digestion in alkaline solution—lactacidogen of Embden—is much reduced. Table III shows a comparison of the analyses of such muscles with those of normal rabbits. The muscle taken for analysis was in each case the gastrocnemius.

Table III.

	Mgm. P. per 100 gm. muscle.			$\frac{B - A}{B} \times 100.$
	A. free.	B. after digestion.	Lactacidogen.	
Normal rabbits—				
1	77	202	125	61·8
*10 minutes	89	—	—	—
2	97	180	83	46·7
*10 minutes	115	—	—	—
Thyroid-fed rabbits—				
1 (1/5 unit insulin)	169	181	12	6·6
2 (dextrose)	167	187	20	10·7

From the normal rabbits one gastrocnemius was taken as rapidly as possible after killing the animal, and the other 10 minutes after death. This served as a control for the effect of any accidental delay in removing the muscles from the rabbits dying of hypoglycæmia. If rabbits, even after long thyroid feeding, were killed by a blow on the head, and not allowed to die of the hypoglycæmia occurring spontaneously or excited by injections of insulin or dextrose, their muscles still showed a normal content of lactacidogen, and rigor mortis appeared only after an interval.

Glycogen and lactic acid.—As in the case of the rabbits dying after large doses of insulin, there appeared to be, in the case of these thyroid-fed rabbits, a direct relation between the occurrence of the precipitate rigor of the muscles and the exhaustion of the carbohydrate reserves. It appeared probable that the low figures obtained for the lactacidogen in the muscles of such animals, nearly the whole of the muscle phosphate being found in the free condition, was due to the lack of glycogen for re-synthesis of the hexose-phosphate. That this was the case, and that the rapid rigor was associated with the failure of this re-synthesis for lack of glycogen, was clearly shown by more complete analyses carried out on rabbits killed by hypoglycæmia, spontaneous or provoked by a small dose of insulin, on the one hand, and rabbits fed with thyroid gland for similar periods, but killed by a blow on the head before the terminal hypoglycæmia appeared. The former went into rigor mortis immediately, the final muscular spasm, as the circulation stopped, being apparently continuous with the rigor; the latter showed rigor only after 45 minutes to an hour.

In animals of both kinds determinations were made of the glycogen in the liver and the muscles; of the free and total phosphate, and from those of the lactacidogen of the muscles; and of the preformed and potential lactic acid of the muscles. From the rabbits dying of hypoglycæmia the muscles taken for analysis were necessarily already going into rigor; those from the animals killed by a blow were taken immediately after death, while still flaccid and living. The results are collected in Table IV, from which it will be clear that the rapid rigor is associated with absence of glycogen, and consequently low lactacidogen value, and the lactic acid present at death, or subsequently developed by incubation, in such muscles is also much lower than the normal; that, on the contrary, the muscles showing delayed rigor retain some glycogen and show normal values for lactacidogen and lactic acid.

Table IV.—Rabbits, after intense thyroid feeding for 18–21 days.

	Dying from hypoglycæmia.		Killed.	
	Rabbit 1.	Rabbit 2.	Rabbit 3.	Rabbit 4.
Time of onset of rigor	Immediate	Immediate	1 hour	45 minutes.
Blood sugar percentage	—	0·023	—	0·111
Liver glycogen percentage	*Nil*	*Nil*	*Traces*	0·3
Muscle glycogen percentage	*Nil*	*Nil*	0·1	0·15
Muscle phosphate in mgm. per 100 grm.				
A.—Free	125	143	78	84
B.—Total	**149**	**165**	**173**	**178**
C.—Lactacidogen	24	22	95	94
Muscle lactic acid—				
Preformed	45	40	150	272
After digestion	89	65	320	468

The rigor of the thyroid-fed rabbits dying of hypoglycæmia was even more rapid in onset than that seen in normal rabbits killed by large doses of insulin, and was also even more rapidly evanescent. The muscles were completely relaxed again after two hours, and sometimes earlier, and if the rigor was forcibly broken, even immediately after its onset, it never returned. In those animals which were killed (R. 3 and 4 of Table IV) the rigor was much later in disappearance as well as in onset, and if broken was to some extent regained. In the animals showing immediate rigor the heart also was found, immediately after death, to be in a condition which, at first sight, suggested intense, permanent systole. The ventricular walls were very hard and obviously dead. The condition was undoubtedly a rigor mortis of the heart muscle, and this was found to contain no glycogen.

A series of rats was also treated with thyroid gland, given in daily doses with the food, and killed at different stages of the feeding. The muscles of the hind limb were taken for glycogen determination, and the onset of rigor mortis in those of the fore limbs was timed. The results are collected in Table V, from which it will be seen that rigor is accelerated when the glycogen content falls below 0·3 per cent., to become immediate when the glycogen is exhausted and death from complete hypoglycæmia occurs. Nos. 1 and 2 were normal controls, and Nos. 3 to 9 had been receiving thyroid for increasing periods.

Table V.—Rats fed with thyroid gland.

All killed except No. 9, which died of hypoglycæmia.

	Time of onset of rigor.	Muscle glycogen percentage.
		per cent.
1	>1½ hours	0·370
2	>1½ hours	0·460
3	>1½ hours	0·310
4	>1½ hours	0·428
5	40 minutes	0·278
6	25 minutes	0·220
7	20 minutes	0·090
8	5 minutes	Nil
9	Immediate	Nil

Hydrogen-ion concentration and rigor.

We have shown that those glycogen-free muscles exhibiting the immediate rigor mortis have less preformed lactic acid and much less capacity for forming it after death of the animal than the normal controls, which would not have shown rigor for some hours. The fact was so difficult to reconcile with the currently accepted view of rigor as produced by acidity, that it seemed desirable to have more direct evidence of the hydrogen-ion concentration actually present in the muscle during the rigor. The recently developed method of measurement with the glass electrode (Kerridge (12)) appeared to be admirably suited, and has already actually been used for the purpose (12A), and Mrs. P. M. T. Kerridge, of the Physiological Institute, University College, London, very kindly made a series of measurements for us on muscles of rabbits dying from hypoglycæmic convulsions produced by large injections of insulin, and on muscles of thyroid-fed rabbits dying from hypoglycæmia.

The rabbits used for the insulin experiments had one sciatic nerve cut two days previously under ether,* and we could compare the hydrogen-ion concentrations in the normally innervated muscles which, having lost their glycogen, went into rigor immediately after death, with that in the denervated muscles, which, having retained glycogen, could be removed for examination still flaccid and irritable up to one hour or more after the death of the animal.

The muscles from the normal side were removed as quickly as possible after death, but it was impossible to obtain them before rigor had set in. Each muscle, whether normal or denervated, was dropped into liquid air and, when frozen hard, was divided into two portions. One was transferred in liquid air to University College, where the determinations with the glass electrode were made; the other was minced in ice-cold 4 per cent. trichloracetic acid for determination of lactic and phosphoric acids. Table VI shows the results.

Table VI.—Rabbits dead from insulin convulsions.

	Muscle.	*p*H.	Mgm. per 100 g.	
			Lactic acid.	Phosphoric acid.
Normal .... Rigor mortis immediate	Gastrocn. Tibial. ant.	6·91 7·04	180 —	141 —
Denervated— Not in rigor. 10 minutes after death .... 70 minutes after death	 Gastrocn. Tibial. ant.	 6·26 6·03	 129 440	 117 130
Normal .... Rigor mortis immediate	Gastrocn. Tibial. ant.	6·67 6·53	54 63	136 132
Denervated— Not in rigor. 10 minutes after death .... 60 minutes after death	 Gastrocn. Tibial. ant.	 5·97 5·92	 176 336	 97 101

The normal muscles were found to be practically glycogen-free.

Table VII gives the results of similar determinations on the muscles of rabbits subjected to prolonged intensive thyroid feeding. In (1) the terminal hypoglycæmia was induced by injecting one-fifth of a unit of insulin. In (2) and (3) death resulted from spontaneous hypoglycæmia. Two determinations on similar muscles from normal rabbits are included as controls.

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* The operations were performed for us by Dr. H. H. Dale.

Table VII.—Rabbits dead of hypoglycæmia after long thyroid feeding.

	Time after death.	Rigor.	pH.	Mgm. per 100 g.	
				Lactic acid.	Phosphoric acid.
1. Thyroid-fed	Immediate .... 90 minutes	Complete .... Passing off	7·0 7·18	66 —	120 —
2. Thyroid-fed	Immediate .... 60 minutes at 37° C.	Complete .... Nearly disappeared	7·29 7·25	25 25	125 125
3. Thyroid-fed	Immediate .... 60 minutes at 37° C.	Complete .... Nearly disappeared	7·23 —	36 23	141 145
4. Normal control	Immediate .... 2 hours at room temp.	Absent .... Absent	6·68 6·45	190 226	72 96

The muscles of 1, 2 and 3 were found to be completely glycogen-free.

Discussion.

The fact that rigor mortis, in the absence of glycogen, may occur with an alkaline reaction was known to and emphasized by Claude Bernard (13). In his "Leçons sur le Diabète," 1877, as evidence that acidity was not the essential cause of rigor, but only a usual concomitant, he states: "Si en effet, on fait périr un animal après avoir fait disparaître le glycogène de ses muscles la rigidité cadavérique se produira quoique le muscle conserve la réaction alcaline."

Bernard also states, concerning crustacean muscle: "Ces muscles (de l'écrevisse) ne contiennent pas en dehors de la mue de matière glycogène. Aussi, après la mort, entrent-ils en rigidité tout en conservant leur réaction alcaline." The statement apparently refers to the muscles of crustacea *just after* the shell has been shed. That the muscles at this stage have no glycogen, and show an alkaline rigor, has been confirmed by one of us (J. P. H.) with Mrs. Kerridge, in experiments which will shortly be published.

Our observations strongly suggest that absence of glycogen is the determining factor of the precipitate rigor which came to our notice. It is presumably also accountable for the rapid evanescence of this rigor, and for the fact that it does not return if broken by force. The fact that a starved animal shows rigor much sooner after death than one which has been fed was also known to Claude Bernard (14). It has been confirmed and investigated by Baur, Kuhn and Wacker (1), who observed that rigor mortis in the muscles of a starved animal may appear 10 to 20 minutes after death, and disappear after six hours. They found that such muscles contain little glycogen.

Caroline Latimer (15), in a study of heat rigor in frog's muscle, found that muscles fatigued by repeated stimulation to the point of exhaustion go into rigor at a lower temperature than resting muscle. The normal condition, in this respect, was restored to the fatigued muscles when they were perfused with dextrose, but not with Ringer's solution or alkaline saline. Latimer concluded that exhaustion of glycogen produced the abnormality, but did not support the conclusion by analysis.

Lee and Harold (16) observed that cats treated with phloridzin and deprived of food exhibited rigor mortis within a few minutes of death. Administration of glucose to such animals before they were killed delayed the rigor. They concluded that absence of carbohydrate favours rigor mortis. Their observations were concerned only with time of onset, not with the strength and persistence of the rigor.

Meyerhof and Himwich (17), in an investigation of rigor in the muscles of rats fed on fat alone, were concerned with the extent of the shortening and the amount of tension developed by the muscles, not with the time of onset. Producing rigor by chloroform, caffeine or heat, they found that the shortening, tension and hardness of the muscles were directly related to the quantity of lactic acid formed. It should be noted, however, that the proportion of lactic acid recorded as responsible for the production of rigor, in some of these muscles poor in glycogen, is far below that which can be found immediately post-mortem in normal muscles, rich in glycogen, which would not begin to show rigor till some hours later.

Much more striking examples of the onset of rigor in muscles with a lactic acid content far below that of the normal resting muscle removed from the circulation, and a pH as high as 7·2, are to be seen in our observations on the glycogen-free muscles of animals dying from hypoglycæmia. It cannot be supposed that this rigor is something entirely different from that occurring in normal muscles, since a series can be produced in which the rigor becomes progressively more rapid, weaker, and more evanescent as the glycogen falls from the normal amount to vanishing point. That when rigor appears, or is induced, its strength is proportional to the lactic acid formed has been made clear by the observations of Meyerhof and his colleagues (17–18). But it is also clear that accumulation of lactic acid does not by itself determine the occurrence of rigor, and that another factor is required, which seems to be the absence of glycogen in the case of the conditions which we have studied. In normal muscle, however, the glycogen is by no means exhausted when rigor sets in, so that the absence of glycogen cannot be regarded as the directly determinant factor. It appears to us that the key to the puzzle may well be provided by recent work from Embden's laboratory (19, 20, 21). It has been shown that, during the anaerobic survival of skeletal muscle, sodium fluoride is a potent activator of the mechanism for re-synthesis of hexose-phosphate (lactacidogen) from the phosphoric acid liberated by previous breakdown and the glycogen present or added. At a certain stage a progressive failure of this synthesis under the influence of fluoride sets in, and it finally fails as the muscle goes into rigor mortis (Deuticke (20)). This has been interpreted as indicating that the change in the muscle colloids, which terminates in rigor, renders the synthesis impossible. In the light of our own observations and those of others, of the immediate appearance of rigor if glycogen is absent, it seems to us that the facts may better be interpreted in the opposite sense, the failure of the re-synthesis of the hexose-phosphate being the determining factor in the onset of the rigor, rather than the reverse. Embden and Jost (21) have shown that in a fatigued muscle the synthetic mechanism is impaired, and the same observation has been made by Andrews (22). In such muscles rigor mortis appears abnormally early, and is again coincident with the rapid decrease of the lactacidogen (Deuticke). When the fatigued muscle is exposed to oxygen, not only is the lactic acid removed, and therewith the characteristics of fatigue, as shown in the classical investigations of Fletcher and Hopkins, but the power of re-synthesizing hexose-phosphate is restored (Embden and Jost), and the rigor mortis again postponed.

All these facts seem to us to point in one direction. Lactic acid by itself will not produce rigor, though the quantity present will determine the amount of shortening and the tension developed by the muscle when rigor appears. For the appearance of rigor, we suggest, there is needed also a rapid disappearance from the muscle of the hexose-phosphate, through failure of the re-synthesis which normally goes on for some time after death of the animal. When the re-synthesis fails for lack of one of the constituents, through exhaustion of the muscle glycogen during life, the rigor appears practically as soon as the circulation stops; but since it occurs in the presence of very little lactic acid, and with no material for the production of more, the rigor is weak and evanescent.

In a normal muscle the re-synthesis fails not for lack of glycogen, but from death of the synthetic mechanism through inadequate supply of oxygen; and under such conditions the rigor, appearing when much lactic acid has already been formed, is strong and persistent.

Why this disappearance of hexose-phosphate should lead to rigor can, at present, be a matter for speculation only. It is conceivable that the hexose-phosphate and the protein of the contractile fibrils do not exist separately, but in the form of a colloidal complex having a certain isoelectric point, and that the cleavage of the hexose not only liberates lactic acid, but produces a protein having a much more alkaline isoelectric point, and therefore sensitive to the small change of reaction produced by the liberation of the acid. It is obvious, however, that much further evidence is required before any theory can be formed as to the true meaning of this peculiar relation of the glycogen and hexose-phosphate to the physical properties of the living muscle protoplasm. It seems proper to suggest, however, that the facts here put forward should be taken into account in any attempt to build a more complete theory, not only of rigor mortis, but also of the much more important phenomenon of muscular contraction, the current conceptions of which have owed so much to the study of rigor.

Summary and Conclusions.

1. The precipitate rigor mortis observed in rabbits, dying after hypoglycæmic convulsions or after prolonged thyroid feeding, is not due to the accumulation of lactic acid, nor to increased acidity of the muscle.
2. The absence of glycogen and the decrease in lactacidogen of the muscle are the determining factors of this rigor.
3. The mechanism of rigor mortis is discussed.

We wish to thank Dr. H. H. Dale for his interest and help in this investigation, and in the presentation of the results. As already mentioned, we are greatly indebted to Mrs. Kerridge for the very important contribution to our evidence made by the determinations which she carried out for us.

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CROONIAN LECTURE:—*The Laws of Muscular Motion.*

By A. V. HILL, F.R.S.

(Lecture delivered May 20, 1926.)

On November 28, 1660, a small body of learned men "according to the usuall custom of most of them" met together at Gresham College in the city of London to hear a lecture by one of their number, Mr. (later Sir Christopher) Wren. "After the lecture was ended, they did, according to their usual manner, withdrawe for mutual converse. Where, amongst other matters that were discoursed of, something was offered about a designe of founding a Colledge for the promoting of Physico-Mathematicall Experimentall Learning." From this "converse," thus recorded in its first Journal-book, arose later the Royal Society. The record adds "Mr. Croone, though absent" (and aged only 27) "was named for Register."

Dr. William Croone, Professor of Rhetoric at Gresham College, Doctor of Medicine of Emmanuel College, Cambridge, and author of a book "*De Ratione Motus Musculorum,*" published in 1664, dying in 1684, left a *plan* for an annual lecture before the Royal Society, on muscular motion, but no provision for it in his will. His widow, who married again, Dame Mary Sadleir, by her will dated 1701, remedied that omission, and directed that one-fifth of the clear rent of the "King's Head Tavern" should be vested in the Royal Society for "the Settling a Lecture for the Advancement of Natural Knowledge," more specifically "A Lecture or Discourse of the Nature and Property of Local Motion, and the application of the Doctrine thereof to explicate the causes and reasons of the Phenomena: every such Lecture and Discourse to be accompanied always, and joined with an Experiment proper for it."* This last provision in Lady Sadleir's will was interpreted by the Society as a means "*to prevent the Lecture from becoming the vehicle of unsubstantial theories.*"

Ward† says that it was to be "of one whole hour, upon the nature or laws of muscular motion . . . accompanied by some experiment, dissection, or other anatomical demonstration, tending to illustrate and explain the subject." The first lecture, upon the motion of the heart, was given in 1738.

A Common Principle in all Vital Motion.

Under the influence of the theory of evolution it has become almost a matter of faith that all types of vital motion, whether in animals, plants or single cells, are ultimately the same in nature. The gentle flowing movement of unicellular organisms, which only the microscope and the kinematograph together can properly represent on the scales of space and time of the imagination; the rhythm and speed of ciliary motion; the magnificent and co-ordinated energy of muscular effort in man and the higher animals; and at the other end of the scale the almost magical quickness of insects; all these are to be regarded as properties of the same fundamental organisation, of the same sequence of molecular changes. Nor is belief in this generalization so difficult, for even in a much narrower category, that of the striated muscles, where one may surely postulate some ultimate common principle in the mechanism, the differences in space and time are scarcely less provoking. The muscle which works the wing of a mosquito many hundreds of times per second, and the one which projects a jumping insect upwards with a velocity not far short of that of a human athlete (after a "take-off" only of the order of a millimetre), are almost certainly based upon the same ultimate mechanism as the muscle of the tortoise's leg, which may on occasions take half a minute to complete a single movement, or that by which the stomach of the crayfish carries out surely, but extremely slowly, the grinding of its food.

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* See 'Report on Medals, etc.,' p. 43, 1834. † 'Lives of Gresham Professors,' p. 320.

Partly for technical reasons of convenience and simplicity, partly because of its intrinsic interest and importance, it is chiefly with this latter category of vital motion, that of the voluntary muscle, that physiological investigation has been concerned. No apology is needed for this limitation. The laws ruling the motion of cross-striated muscle, and the molecular organization and changes underlying it, once laid bare, will be found to provide the key to many other of the phenomena of life. There can, however, be no doubt—recent work, indeed, has amply proved—that the investigation of other forms of movement, in cilia, in amœbæ, in involuntary muscle, can confirm and widely extend the knowledge gained from the more specialized study.

The evidence of a common principle in all animal movement has been chiefly of a circumstantial or of an observational character. From the point of view of individual and racial evolution, cells having a similar ancestry or a common manner of development would be unlikely to employ different mechanisms for the same end. Recently, however, evidence of a different type, chemical and physical rather than biological, has accumulated, that such a common principle does in fact exist. We will consider some examples of this evidence.

Let H be the heat produced in the "initial" stages of an isometric contraction resulting from a stimulus of short duration in a muscle of length l, which at the same time develops a tension T throughout its length; the ratio H/Tl, being of no dimensions, is independent of the units employed and might conceivably have any value, positive or negative. In the leg muscles of frog, tortoise or toad, we have known (1, p. 508) for some time that the value of this ratio is uniformly about 0·2, varying perhaps from 0·1 to 0·4: in the jaw muscles of the dogfish we find exactly similar results: while recent experiments by my colleague, Lehnartz, on the smooth muscle of the foot of the common sea-mussel *Mytilus* have given values lying within the same range. It is a long way in evolution from the striated muscle of frog, toad, tortoise or dogfish to the smooth muscle of Mytilus: yet apparently there is a mechanism in each adhering sufficiently closely to a common plan to give us the same absolute value of our ratio.

Again, let us consider the manner of utilization of the oxygen which the cell requires to provide it, by oxidation, with the energy it needs for movement. It was possible—indeed, it was long supposed—that in muscle this oxygen was used during the contraction itself in some kind of explosive chemical change which induced the motion. This view is certainly wrong. [See (2) p. 56.] It was disposed of by Fletcher and Hopkins in another Croonian Lecture (24). Activity can occur in the complete absence of oxygen. Oxidation is indeed necessary,* but only subsequent to activity, and in order to restore the muscle to its previous state, to provide the energy necessary to reverse the initial chemical breakdown.

What is true of the voluntary muscles of all the animals investigated, ranging from man to frog, is equally true of ciliated cells, whether from the œsophagus of the frog (Engelmann (3)) or from the gills of Mytilus (J. Gray (4)); and it is true, as Mr. C. F. A. Pantin tells me, of the movements of marine amœbæ, which can continue their slow progression, by the protrusion of an anterior pseudopodium, for an hour or more after they have been deprived of oxygen; finally, indeed, they stop but recover on changing to oxygenated sea-water. Moreover, recent work by Evans (5) on the properties of smooth muscle makes it almost certain that in this also oxygen subserves the same function of recovery. Movement, therefore, without oxidative change, followed in the presence of oxygen by restoration, seems to be a general property of all contractile cells.*

The force exerted at any moment by a contracting muscle is a function, not only of its length at that moment, but of the speed at which it has attained that length (6). As we shall see later, the alteration of force exerted with velocity of shortening is a fundamental characteristic of muscle, varying with the function which it has to fulfil, and supplying an important clue as to its ultimate physico-chemical mechanism. The relations found, though differing immensely in their time factors, are the same in character in the cross-striated muscles of man, frog, and tortoise as in the smooth, slowly reacting muscles of marine invertebrates. Without some common principle the chance of such a concordance would be small. Examples, indeed, might be multiplied: the effect and antagonism of ions; the mutual transformation of glycogen and lactic acid; the influence of acids and alkalies. There can be little doubt that in discussing the processes of muscle, as Dr. Croone planned, now 250 years ago, we are dealing with a common principle which underlies most, if not all, of the forms of motion manifested by living cells.

Methods of Investigating Muscle.

Apart from its capacity for growth and self-repair, and for adjusting (as it grows) its physical and chemical characteristics to the needs of its owner, a muscle is essentially a mechanism for transforming chemical into mechanical energy. There are, therefore, three chief ways in which it may be studied: the chemical, the mechanical and the thermodynamic, each essential, each possessing advantages and difficulties of its own. Nothing but a *chemical method* can reveal the actual substances taking part; its fundamental difficulty is that to apply any ordinary chemical treatment to the inside of a muscle fibre means the inevitable destruction of the latter. Essential information has been gained by chemical study, but its scope is limited: for the finer analysis of the mechanism, finer tools are necessary. The *mechanical response* of muscle has been investigated for generations, yet to show how the growth of one science influences another, one of the most striking of its properties—its apparent "viscosity"—is only now beginning, under the influence of modern physical chemistry, to have its pertinence realized. The *myothermic method*, dealing with the energy exchanges, has the advantage of measuring in absolute units, without injury to the structure, an important characteristic of the internal changes which take place during activity; and by reason of the fineness of its technique it can deal adequately with quantities which at present biochemistry cannot even detect. Its limitation—that of any thermodynamic method—is that it can provide only the outline and not the details of the mechanism.

Chemical Studies of Muscle.

When a muscle is excited it responds with a contraction and its temperature rises. If the stimulation be continued it gradually fatigues; lactic acid appears within it and an equivalent quantity of its glycogen is lost, while certain changes, as yet imperfectly understood but obviously important, occur in its inorganic phosphate content.* None of these events involves the utilization of oxygen; the processes of contraction and relaxation are clearly independent of any provision of energy by contemporary oxidation. In the presence of oxygen activity is followed by recovery; lactic acid disappears, glycogen reappears, carbon dioxide is produced, heat is liberated, and the muscle is restored to its previous state of readiness to respond. The substance oxidized within the muscle fibre, to provide the energy for the endothermic processes of recovery, is apparently carbohydrate, one part of this being burnt in effecting the removal of four parts of lactic acid. There is no evidence that the isolated muscle can use for its oxidation any other substance than carbohydrate. Fat may be employed by the body as a whole to provide the energy for muscular exercise, but presumably only after such transformation as brings it to the muscle in the form of carbohydrate, for use in the recovery mechanism. What the nature of that mechanism may be we do not know yet; there lies one of the major problems of present-day biochemistry, a problem of extraordinary importance since the reaction by which lactic acid is formed from carbohydrate in the absence of oxygen, and re-synthesized into it in the presence of oxygen, appears to be well-nigh universal, and not merely a characteristic of the muscle cell.

A general outline of the processes involved in this "recovery" is provided by the study of the heat production with which it is associated; this gradually rises to a maximum, then more gradually declines; its rate is largely affected by the hydrogen ion concentration and by the quantities present of the reacting bodies, suggesting a bi-molecular process of some kind. The heat liberated in recovery is about one and a-half times, while the energy retained in a latent chemical form in the re-synthesis is about equal to, the total energy set free in the initial breakdown. If these ratios were always the same, one might be tempted to look for some invariable sequence of chemical changes effecting, in the presence of oxygen, the transformation of lactic acid to glycogen. In frog's muscle the ratio of recovery heat to initial energy is variable, but scarcely outside the limits of experimental error. In tortoises, however, as Mr. Hartree tells me the ratio may commonly be as great as 2·5, showing a much lower "efficiency of recovery": it may vary from 1·0 to 3·0. If the "efficiency of recovery" can alter in this way, it would seem unlikely that any regular sequence of chemical events is in the back-ground; it would appear rather that we have two separate factors, a machine running on the energy supplied by oxidation, itself driving a second machine by which the re-synthesis is effected. To take a simple engineering analogy, we have a combustion engine driving a dynamo which is re-charging a battery of accumulators. With such an arrangement the efficiency of restoration might well vary according to the nature and condition of the machines.

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* For references see (2), pp. 82 and 111, or (7), p. 101, &c.

Do the chemical changes which we know to occur in anaerobic activity account completely for the mechanical response? or are there other chemical reactions of which, as yet, we know nothing that form an integral part of the picture? The energy necessarily associated with the transformation—which undoubtedly occurs—of glycogen into sodium or potassium lactate, supplies all—or nearly all—the heat which the muscle liberates in prolonged anaerobic activity; any other reactions, therefore, which accompany contraction, must either be of small importance from the energetic standpoint, or must be completely reversed. Now it can be shown that the single twitch does not differ sensibly from prolonged activity, in respect of its energy relations. Taking Matsuoka's (8) value [108] for the "isometric coefficient" of lactic acid, and Meyerhof's (9) [390 cals.] for the heat associated with the production of 1 gm. of lactic acid, both estimated for *prolonged activity*, we may calculate that H/Tl in frog's muscle is 0·15; direct observation of H and T in *single twitches* gives about 0·20; a very fair agreement. Moreover, recent experiments by Furusawa and Hartree have tended to show that in the complete absence of oxygen there is little liberation of energy after relaxation is complete. In contraction and relaxation, therefore, as in prolonged activity, the chemical changes which are known to occur supply all—or nearly all—the energy which is liberated. We must look, therefore, either for reactions which are completely reversed by the time that relaxation is complete, or for such as involve a negligible energy-exchange.

Now it is obvious that the mechanical processes of contraction are reversed in relaxation, and, therefore, also the physico-chemical events immediately underlying them; of these, in detail, we are ignorant, though in a general way it would seem probable that they involve the formation and disappearance of a new molecular pattern, analogous perhaps to a system of liquid crystals (Garner (10)), in the protoplasm of the muscle. Those processes which go full circle do not come into our balance-sheet; of others suggested there is only one, the mysterious one involving phosphate. It is probable that lactic acid comes only indirectly from glycogen, through an intermediary hexose-phosphate (Embden); either therefore the transformation of hexose-phosphate into lactic acid and phosphate goes on with little liberation of energy—or the hexose-phosphate is completely re-formed from glycogen by the time that relaxation is complete. Both, indeed, are very probably the case.

If there be a complete absence of glycogen—as in starved animals fed with thyroid, after insulin convulsions—then the hexose-phosphate cannot be reformed and a curious thing happens (Hoet and Marks (11)) : a form of rigor sets in characterized by the absence of acidity and lactate. It would seem therefore that the hexose-phosphate is somehow associated with the structural elements of the muscle : when it breaks down these are left momentarily " unprotected " and exposed to the action of the acid, which works upon them causing the mechanical response : the hexose-phosphate is then re-formed, the structural elements return to their previous state, the lactic acid becomes sodium lactate, and the muscle relaxes. In the complete absence of glycogen no such return is possible, for the hexose-phosphate cannot be re-formed, relaxation does not occur, and a state of rigor—or rather possibly of unreversed contraction—sets in. So far, therefore, as our present knowledge goes, of lasting changes associated with the first and anaerobic phase of muscular activity there is only one, the transformation of glycogen into lactate ; but within the full course of activity—contraction and relaxation—there are other processes, one involving the breakdown and re-formation of hexose-phosphate in some organic connection with the structure of the muscle, a second, the creation and disintegration of a molecular pattern associated with, and causing, the mechanical response.

Myothermic Studies of Muscle.

Perhaps the most important of the conclusions derived from the study of the heat-production of muscle is that the processes of activity can be split, very sharply and without ambiguity, into separate phases. There is the " initial " process, complete maybe in a fraction of a second, independent of the presence of oxygen, associated with contraction and relaxation and with a rapid evolution of heat : and there is the " recovery " process, requiring 15 minutes or more for its fulfilment absolutely dependent on the presence of oxygen, associated with no visible mechanical changes, and accompanied by a slow and gentle heat-production. A similar division into " initial " process and oxidative recovery seems to be possible also in the case of nerve (12). These two phases we have discussed already, in connection with the chemical aspect of the problem. Further analysis, however, is possible. The initial process itself can be split, again without ambiguity, into separate phases : the analysis is not easy, the quantities are small—the whole rise of temperature involved is only a few thousandths of a degree—and the events follow one another with great rapidity; the experiments, however, of Mr. W. Hartree (13) at Cambridge, have revealed in beautiful clearness the outline of processes of which, at present, we can only guess the chemical basis. Immediately on stimulation there is a large and rapid evolution of heat; this, in a contraction of short duration, represents about half the "initial" energy: it is complete long before the moment when the maximum tension is attained; then follows relaxation, accompanied by the evolution of the other half of the "initial" heat. In a prolonged contraction there is another equally obvious phase, that associated with a constant rate of heat-production, lasting as long as the contraction is maintained. The time is not ripe for a detailed allocation of chemical processes to these several phases. We know, perhaps for certain, the total resultant of all the chemical events associated with activity. Such knowledge, however, omits all mention of the processes which go on in contraction and are reversed in relaxation; these also we must find some means to study, aided by their thermodynamic outline, encouraged by the certainty that they are there.

The maintenance of a contraction is accompanied by a continual degradation of energy: just as the pull of an electromagnet can be upheld only by passing a current continuously through its coil. Muscles of great intrinsic rapidity quickly fatigue during the maintenance of contraction; those designed for lower speeds are less readily tired thereby. In a general way a simple dimensional principle exists. Each muscle has a time-scale of its own; employing its own unit of time, which is proportional to the interval occupied in a single twitch—each muscle maintains its contraction with the same economy, each becomes fatigued at the same rate. The time-scale of a muscle is not absolutely fixed; nor probably does recovery, but only the initial process, adhere to it: it may vary according to conditions, *e.g.*, with temperature; even with such qualifications, however, each type of muscle has, in a general way, its characteristic scale of time which determines not only one but a whole set of interrelated physical, chemical and physiological phenomena. From the biological point of view, these functional differences between different muscles are of extraordinary interest; the variations are so wide, yet the adaptations to the needs of the animal so exact. The theory of dimensions may be invoked to tell one what, in an ideal world, one might expect of an animal's athletic capabilities; and behold! by an adjustment of the time-scale of his muscles Nature has frequently anticipated one's dimensional reasoning. How—from the physico-chemical standpoint—this adjustment of the time-scale is made is a question which we will discuss later.

The Effect of Doing Work upon the Total Energy Exchange.

The study of the heat production of muscles has thrown light upon many details of their behaviour : the action of drugs, and ions ; the effects of temperature and extension ; with such details, however, this is not the occasion to deal. There remains, however, a set of facts of obvious and first-rate importance (14), (15) which—to the dismay of their discoverer—(having a certain interest in them for they were studied first in the cellar of my house) I have ventured to name the "Fenn effect." When a frog's muscle contracts and relaxes, without shortening, it liberates a certain amount of heat ; if it be allowed to shorten and do work, it liberates extra energy about equal to the work it does ; in whatever way one lets it work it liberates extra energy in proportion to the work. If, on the other hand, it be forced to lengthen instead of allowed to shorten, it liberates less energy, in proportion to the work that is done upon it. It is easy to conclude that in the machinery of the muscle there is some regulatory arrangement, adjusting the output of energy to the work which must be performed ; governor mechanisms of this type are common in engineering. Such a conclusion, however, is little but an expression of the facts themselves. What is the nature of this governor mechanism ?

When a muscle is excited at constant length its tension rises and it reaches temporarily a state in which (like any stretched elastic body) it possesses mechanical potential energy, which it can use for doing work if it be allowed to shorten. The full amount of work cannot be done—owing to the "viscosity" of the muscle substance—unless the shortening be slow, unless, that is, the conditions be, in the thermodynamic sense, reversible. Now, Hartree (16) found—in confirmation of Fenn—that in a frog's muscle allowed to shorten, doing or without doing work, the *heat* set free is the same as in an isometric contraction, an extra amount of *energy* being liberated to provide for the work, if any, performed. He ascertained, however, that when *shortening* was allowed there was 15 per cent. extra heat in the first (contraction) phase and a corresponding deficit in the second (relaxation). When *work* was done, the energy to supply it was liberated of course in the first (contraction) phase. The matter may be expressed most conveniently in a table ; the work naturally is arbitrary and depends upon conditions ; in Hartree's experiments it had a mean value as shown.

—	Contraction.		Relaxation.	Total.	
	Heat.	Work.	Heat.	Heat.	Energy.
Isometric	50	—	50	100	100
Shortening with small load	63	3	35	100	103
Shortening with large load	65	26	35	100	126

Apparently, therefore, the process of shortening, as such and independently of the work done, causes a shift in the production of heat between the two initial phases; while the doing of work results in a greater liberation of energy.

It is difficult to see any simple explanation of these, and of other even more complex phenomena discovered by Fenn and further investigated by Azuma (17). There are too many factors at work. The resting muscle shows vividly the thermo-elastic phenomena (18) described by Kelvin, which are the thermodynamic converse of the expansion (or contraction) with rise of temperature. Why not, therefore, the active muscle? These thermo-elastic effects are reversible in a body in equilibrium and cancel out in the complete cycle; but who can say how far they balance one another when the body which shortens (in contraction) is really, in many physical respects, a different one from that which lengthens subsequently (in relaxation)? Then there is the mechanical potential energy of the active muscle which may be, but is not necessarily, dissipated quantitatively as heat in relaxation. The system is too involved; we must choose a simpler case for study.

It was known to Blix and to Fick (19, pp. 23–25) that while an active tetanised muscle is being stretched the tension-length curve lies higher than that found while it is being allowed to shorten. Fick, unable to understand this very surprising phenomenon, did what was rare indeed for him, he relapsed (if I may use the term) into a "physiological" explanation; he argued that the stretch of the muscle increased its excitability and caused it to respond more vigorously to the stimulus, while shortening presumably did the reverse. This was clearly the contrary of what one would expect from Fenn's work, and Wyman (20) has recently applied to it a definite test. Taking a muscle maintained in constant maximal activity by a tetanus, releasing it to do work only while its contraction was fully maintained, and allowing it to shorten (or causing it to lengthen) at a definite and constant speed—conditions far simpler to control and understand than those of Fenn and Azuma—he studied the total energy liberated, as an index of its physiological activity. In the result Fick's hypothesis was not confirmed; the response of the muscle allowed to shorten, as estimated from its liberation of energy—and there could not well be a better way—was not less, but considerably more, than that of the muscle caused to lengthen. We shall be dealing later with the real cause of the phenomenon—the so-called "viscous-elastic" properties of muscle—but for the moment we are concerned with the light which Wyman's results may throw upon the mechanism of the "Fenn effect."

It has long been tacitly assumed that the mechanical potential energy undoubtedly possessed by the active muscle is degraded quantitatively into heat in relaxation. The discovery that a large proportion of the initial heat is set free in relaxation, added countenance to, though it did not necessarily establish, this assumption.

The area of the tension-length curve of the active muscle, which—if there were any rhyme or reason in regarding the muscle as an elastic body—represented the real potential energy of the active muscle, might be larger even than the total energy given out when it contracted isometrically (21). Some of this potential energy—if not used for doing external work—must presumably be reabsorbed, when the muscle relaxed: employed perhaps in effecting a chemical re-synthesis of bodies broken down in contraction. Now there is no doubt that, in recovery, chemical re-synthesis does go on, under the influence of the chemical energy liberated by oxidation in that phase: if such may happen why not also during the disappearance of the mechanical energy of the excited muscle? Of all forms of energy the mechanical is the most readily available for general purposes: if unutilised for doing work might not an economical providence have designed that it should be used up, in whole or in part, for the restoration of the *status quo?*

When a muscle, as in Wyman's experiments, has shortened and done work, it has lost some of its potential energy: consequently there is less to dissipate—or to reabsorb—in relaxation. Conversely, when it has been stretched and has had work done upon it, it has gained potential energy: there is more to get rid of in relaxation. Let us suppose that the energy set free initially—as potential energy and as heat—is the same in either case, but that x per cent. of the potential energy is reabsorbed in relaxation: then if there be less potential energy present when relaxation sets in there will be less reabsorption, and a greater resultant liberation of total energy: while if there be more potential energy at this stage there will be greater absolute reabsorption, and a smaller resultant liberation of total energy in the whole cycle. Wyman's results clearly fitted this hypothesis, and a quantitative treatment gave him the value of about 33 per cent. for x. Suggestively enough, this is not far from the ratio (40 per cent.) of the energy known to be absorbed in endothermic processes during recovery to the total energy set free by oxidation in that phase.

We have come therefore to a pertinent hypothesis on which to base further experiments. The active muscle is an elastic body possessing potential energy, measurable from its tension-length curve. If it be allowed to shorten it will do such external work as the conditions permit. The balance of its potential energy goes in relaxation in two ways—(*a*) in driving endothermic processes to restore it to its state before contraction (1/3), and (*b*) by dissipation into heat (2/3). Such restoration as is not effected in relaxation is then completed, at the expense of chemical energy liberated by oxidation, in the prolonged phase of recovery.

" *Viscous-Elastic* " *Phenomena in Muscle.*

One aspect of the laws which govern muscular motion—the so-called viscous-elastic phenomena—I have mentioned on various occasions already: with these I must now more specifically deal. If Fick's hypothesis be incorrect, if the physiological response of the muscle undergoing stretch be not more but less than that of the muscle allowed to shorten, why is it that the tension at any given length is so much greater during stretch than during shortening? The answer to that question, providing—as I believe—a most important clue to the physico-chemical nature of the contractile process, requires some preliminary description and discussion.

If the process of contraction is to be regarded as a change-over from the elastic properties of the resting to those of the active muscle—and both show obvious signs of the physical characteristics associated with elasticity—then the maximum work which a muscle can do in shortening may obviously be derived from a curve relating tension to length. There is a difficulty in the notion that a muscle, on stimulation, merely changes its "natural length" and its "elastic coefficient" from those characteristic of rest to those characteristic of activity, viz., the fact that the physiological cost—as measured by the heat-production—of maintaining a maximal tension varies with the length at which the tension is maintained. The muscle is not simply one definite elastic string during rest, another during activity. It is obvious, nevertheless, that the maximum work *must* be equal to the area of the tension-length curve, however complex the real elastic nature of the contracting muscle. Now it has long been known that it is difficult, in practice, to cause a muscle to do work even approximating, in amount, to the area of its "indicator diagram." This is due to various causes, partly to the conditions of loading—that is, to inadequate means of collecting the work—but partly to the speed at which, under ordinary circumstances, the shortening of the muscle occurs. It was shown by Fick (19), many years ago, that work nearly equal to the area of the curve can actually be obtained, provided (*a*) that the muscle be kept in prolonged contraction, (*b*) that adequate technical means for obtaining the work be employed, and (*c*) that these be so adjusted that the process of shortening takes place comparatively slowly. There was no doubt of the validity, under certain circumstances, of the conception of maximum work when applied to an active shortening muscle; the interest lay in the study of the complicating factors.

Of these factors, one only, the speed of shortening, is really of more than technical interest: the experimental difficulties of obtaining and measuring the work have gradually been resolved, and it is now possible, by means of methods recently elaborated by Levin and Wyman, to obtain, with precision and ease, the work actually done by a muscle shortening between any specified limits, at any given and constant speed, simply as a tension-length curve. I will omit the historical antecedents, ranging over several years of experiment, and simply quote from the results which they have permitted me to use. A muscle is excited by a maximal tetanic stimulus, its tension rises, and when that has attained its maximum the muscle is allowed to shorten between given limits at some constant speed (adjusted by a dash-pot with a needle-valve); the tetanus is maintained until shortening is complete, when the muscle is allowed to relax. As it shortens it does work, pulling upon a device which automatically writes a tension-length curve upon a smoked-glass surface. A set of typical records, copied and superimposed, is shown in fig. 1. The work done, calculated from the area of the curves, may then be plotted against the speed of shortening, and we obtain a relation shown in fig. 2. This relation may be determined for any given muscle with great accuracy, and is a characteristic of the particular muscle employed: in respect of the scale of time it

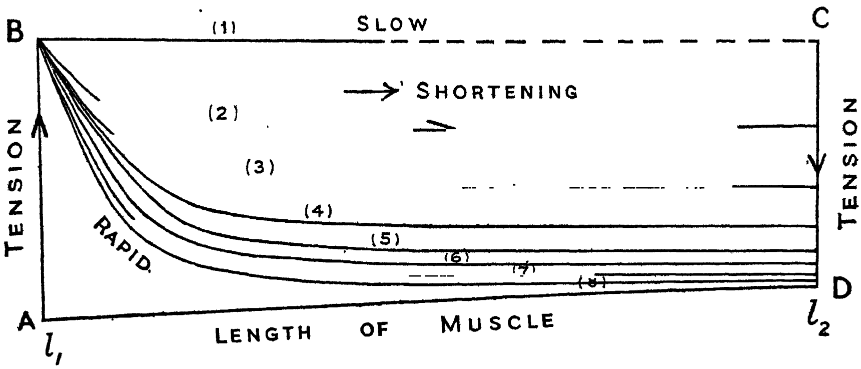

FIG. 1.—Tension-length curves of muscle shortening, during tetanus, at various constant speeds. Muscle stimulated isometrically at length l_1 till maximum tension AB developed; then allowed to shorten at constant speed to length l_2; tetanus discontinued when shortening complete; isometric tension at l_2 = DC. Speeds (relative): (1) 0·083, (2) 0·59, (3) 1·31, (4) 2·27, (5) 3·79, (6) 5·0, (7) 6·58, (8) 10·85. (Levin and Wyman.)

varies enormously—hundreds or even thousands of times—from one muscle to another; otherwise, so far as our present knowledge goes, it is invariable

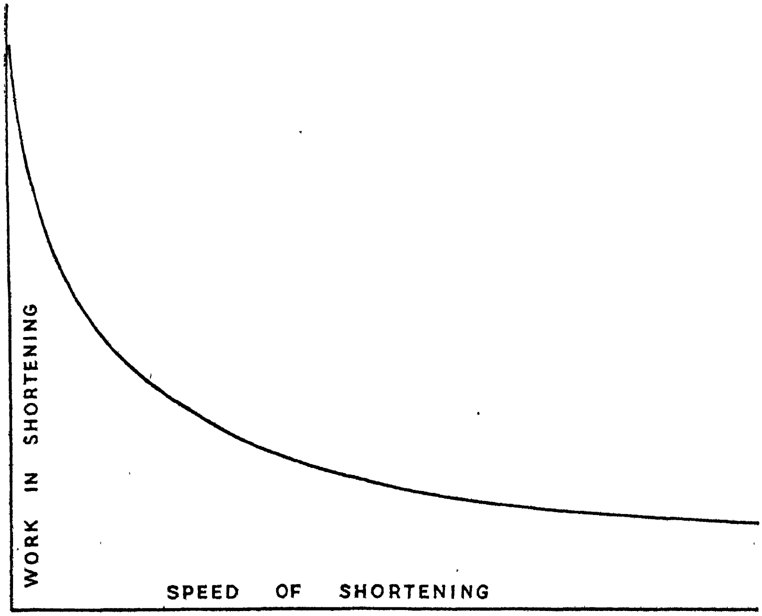

FIG. 2.—Work done in shortening (calculated from areas of Fig. 1) as a function of speed; both in arbitrary units. (Levin and Wyman.)

in type in all contractile tissues. In quantitative terms, the relative speed of shortening $\left(\frac{1}{l}\frac{dl}{dt}\right.$, where l is length and t is time$\left.\right)$ at which the work is reduced to one-half of its theoretical maximum, varies between the widest limits as we pass from, say, the wing muscles of insects, *via* frog, man and tortoise, to, say, the smooth muscles of marine invertebrates. In this quantitative factor we have something typical of the individual muscle; in the relation itself something of a general character. It is clearly of importance to enquire more closely into each.

From the point of view of dealing with these properties mathematically one wished to ascertain the effect of an element of shortening δl carried out instantaneously: for of such elements occurring at regular intervals δt any finite shortening at any finite speed may be regarded as composed. Clearly in the effect on the muscle of a small instantaneous release we might expect to find the simplest expression of the phenomenon in question. Such reasoning led Gasser and me (6) to study the—as it proved—astonishing results of allowing a muscle undergoing an isometric tetanus suddenly to shorten a small distance. Were one to carry out such an experiment with an elastic spring one would find—apart from a few vibrations rapidly damped out—merely a drop from the tension corresponding to the initial to that corresponding to the final length. Not so with the muscle. The tension fell towards zero, and then rose again, but only slowly, to its final equilibrium value. We noticed, moreover, that the curve along which it rose after release was always, within the limits of error of such observations, identical with that along which it rose initially when stimulation began. Recently I have had the opportunity of carrying out similar experiments—which technically are much simpler—upon the slow body muscles of Holothuria. Another series has been performed upon the voluntary muscles

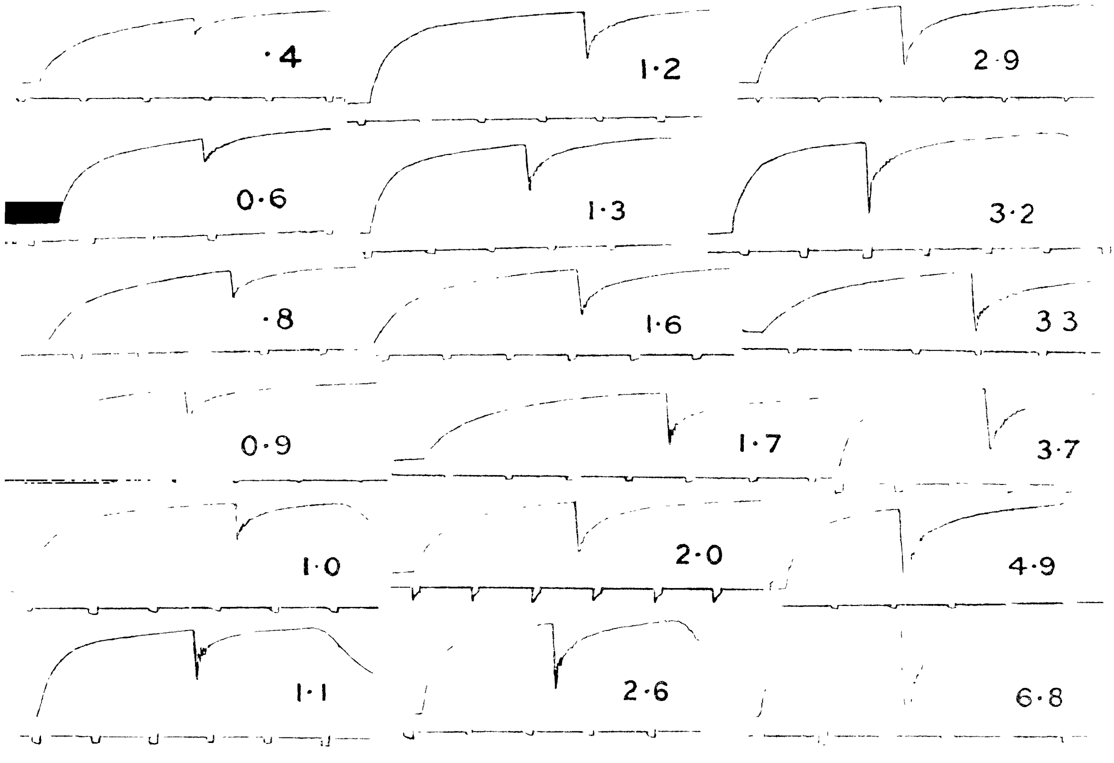

Fig. 3.—Isometric tetanus of biceps cruris of tortoise (65 mm. long, room temperature) with quick release during the stimulus. The numbers on the curves represent the extent, in millimetres, of the quick release. Time marks, seconds.

of the tortoise, of which some typical results are shown in fig. 3. These amply confirm the experiments I made with Gasser—it is clear that the process of redeveloping a tension after sudden release is similar in its time relations to the process of developing a tension when stimulation commenced. If so, one cannot but suspect that the processes themselves are the same and not merely their velocities. It is interesting to see where this hypothesis leads.

At this stage it is necessary to introduce a physical analogy to the phenomena we are studying, the analogy of an elastic system possessing considerable viscosity.* Imagine a closed elastic tube (*e.g.*, of thin rubber) filled with a material of high viscosity (*e.g.*, gear oil or pitch), and connected with any of the arrangements we have employed for recording the work, or the tension, of muscles. The tension-length curve of such a " viscous-elastic " system will depend upon conditions : only if these be reversible, in the thermodynamic sense, *i.e.*, only if each length be maintained long enough for the tension to become constant, does the curve truly represent the elastic properties of the body : otherwise it depends (*a*) upon the coefficient of viscosity ; (*b*) upon the speed at which each length is attained ; and (*c*) possibly also upon previous speeds at neighbouring lengths. The general characteristics of such a " viscous-elastic " system are obvious on simple dynamical principles : they can be expressed in the form of the tension-length curves of a process of shortening carried out at various speeds. It is clear that the work done in shortening must diminish with increase of speed, the difference between the theoretical maximum and the actual performance being dissipated as heat in changing the form, at a finite speed, of the viscous material inside the elastic tube. It is equally clear that the effect of a quick release must be to cause a large sudden decrease of tension, followed by a subsequent slower rise occurring at a rate depending upon the constants of the system. What the behaviour of the model would be was so obvious that it was perhaps unnecessary to make trial of it : Gasser and I, however, as in duty bound, actually constructed such models, which on trial behaved as it was obvious they must, and perhaps may have helped to convince —or amuse—our friends.

Now a resting muscle, even a dead one, shows all the characteristics of a " viscous-elastic " system : the well-known phenomena of " after-extension " : the irreversible thermal effects of extension or release (18). The apparent " viscosity," however, of the active muscle is far greater than that of the resting one, and it was necessary to suppose that the "viscosity," as well as the "elasticity," of the muscle is largely altered by stimulation. On this supposition, apart from the rise of tension on stimulation which obviously cannot be expected of the model, all the phenomena we have been discussing in muscle can be found in an elastic tube filled with a viscous material, or in some similar viscous-elastic system. One was obviously on the track of some real and important property of the physico-chemical mechanism of the contractile process.

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* [Added in proof. Sir William Bragg has kindly called my attention to the existence of similar viscous-elastic properties in naturally occurring fibres, *e.g.*, of cotton or wool. These have recently been widely investigated, in connection particularly with the textile industries. See, for example, Shorter (25).]

Was one, however, simply to accept the model, and to suppose that on stimulation a new elastic network was constituted, holding between its meshes a viscous fluid, which when the muscle changed its shape had to be forced to flow into a new configuration ? At first one did so, perhaps not really believing one's own hypothesis, but confident that for awhile it could be tested and not found wanting, that it could predict results whose verification, or otherwise, would carry one further into the problem. The extreme differences, however, which exist between different muscles, in respect of the speed at which they can shorten and do work, and the way in which the rate of development of an *isometric* response runs parallel with that speed, made one become doubtful whether simple viscosity was involved. There are many physico-chemical effects which show an equal dependence upon speed. If one were to assume, in a general way, as Garner (10) has done rather more specifically, that the onset of activity in a muscle is accompanied by the formation of some new molecular pattern, either in the surfaces or throughout the volume of the fibre, then one might equally well explain the facts. The equilibrium condition is not always instantaneously reached in a film of molecules packed upon a surface, nor probably is the spatial lattice of a crystalline liquid formed in a moment. The speed of gelation of a protein, involving the arrangement of its molecules in some kind of regular order, may be slow and largely altered by minute changes in its chemical constitution. In some such way, rather than by invoking simple physical viscosity, I think one must proceed. Viscosity in a sense it is : not gross, but molecular : molecules do not come instantly into balance with one another, either physically in a surface or a spatial lattice, or chemically in a reversible equilibrium. This slowness of attainment of a final state, involving molecular "viscosity," is a common property of many physico-chemical systems : if such a system be employed for doing mechanical work, this hysteresis will manifest itself as ordinary "viscosity."

We may return now to the consequences of the "quick-release" experiment, viz., the fact that the speed of re-development of a contraction after release is identical with that of its original development when stimulation began. At rest the molecules of the muscle fibre are in some kind of equilibrium (A) with one another: that this equilibrium may involve, at any rate in striated muscle, some spatial organization analogous to that of a crystalline liquid, is suggested by the presence of doubly-refracting bands visible under the miscroscope. As soon as stimulation commences the equilibrium changes from (A) to (B), one factor in the equilibrium being the length of the muscle, another the force which it exerts. The attainment, however, of (B) from (A) is not instantaneous: it proceeds gradually, at a rate depending upon the properties of the molecules involved. Imagine it completed and the muscle then suddenly released. The balance is upset: the molecules are now no longer in proper relation to one another for the shorter length: their spatial patterns must be rebuilt in a different external form. The equilibrium in fact must pass from a state (B) to an analogous but different state (B′): until that has been done the force exerted cannot attain the steady value characteristic of the final length.

We are dealing with a very general property of equilibria. The time-curve along which a true equilibrium is attained, in a system either without inertia or sufficiently damped by friction, is always analogous to the rise of an isometric contraction in a stimulated muscle: while, if by any means the position of equilibrium is altered suddenly, the attainment of the new equilibrium proceeds along a similar curve. The phenomena, therefore, which we have found in muscle are old and very familiar: only, viewed aright, they bring the muscle into a very definite physical category.

To take a more specific analogy, let us consider the packing of molecules in films on the surface of water. Many substances, according to N. K. Adam (22), show no detectable hysteresis in their compression curves. The molecules have reached their equilibrium pattern in the surface, at a given average area per molecule, before an observation of the surface pressure can be made. Other substances, however, do very definitely show such hysteresis. Mr. Adam informs me that he has recently discovered a striking case in films of hydrolecithin. If the area be suddenly decreased the pressure may at first be several dynes per centimetre greater than the final pressure, to which it falls in several minutes. The fall is approximately exponential in the later stage, being more rapid at first. In the same way, if the area be suddenly increased the pressure takes a long time to rise to its final value. Equilibrium, however, can finally be attained from either side. He informs me also that there is probably slight hysteresis even in the case of myristic acid, and that this is most marked where the "expanded" curve begins to turn into the "condensed." Still another example is given in fig. 4* (from Adam and Dyer). The changes with hydrolecithin are exactly similar to those which occur with muscle following

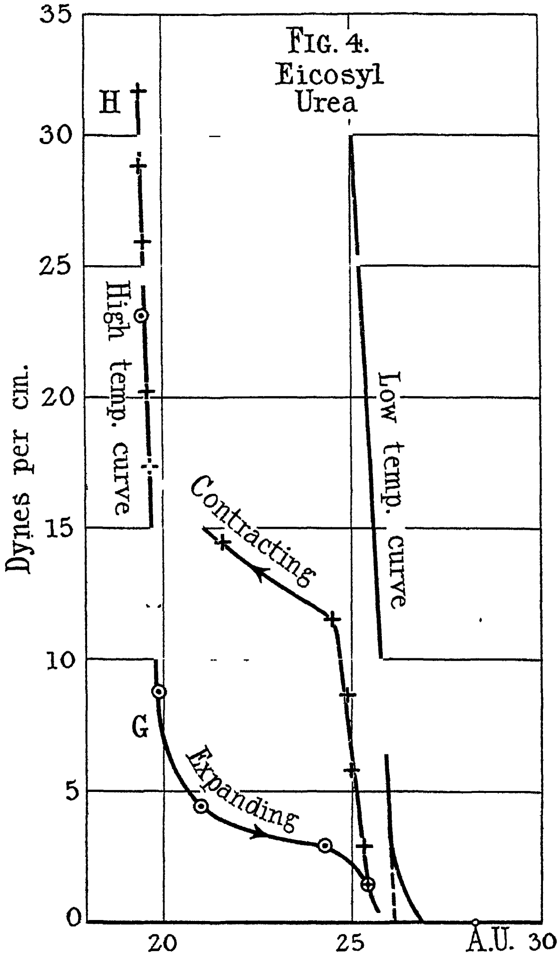

FIG. 4.—Compression curves of eicosyl urea, in a monomolecular film on the surface of water, in the neighbourhood of the transition temperature. Right-hand curve, below 25° ; left-hand curve, above 35°. Curve with arrows, at 30·5°, showing hysteresis ; the area of the hysteresis loop shown depends upon the rate at which the compression is applied or removed. (Adam and Dyer, 1924.)

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* Mr. Adam informs me that the urea derivative employed in making this curve, below the transition temperature, pack with their heads forming a two-dimensional solid structure of area about 25 × 10^{-16} sq. cms., so that their long chains are kept slightly apart. At the transition temperature (between two different types of packing) this two-dimensional solid, which extends only over a short length of the molecule, breaks down, so that the chains can come together. The hysteresis in this case is due simply to the fact that the formation, or the break up, of the solid structure takes time.

either a quick release or a quick stretch, and there can be no doubt that the same thing would happen with any substance showing hysteresis in its compression curve.

In admitting the closeness of the analogy one does not assert that the contractile process is necessarily concerned only with surface forces : what is true of two-dimensional patterns, or rearrangements, must be true also of those requiring three dimensions for their manifestation. The formation of jellies or crystals is often slow : the rate of production of a blood clot may vary widely with minute changes in the constitution of the plasma. The formation of such a structure during stimulation, its disintegration during relaxation, its gradual tightening up, as a preliminary to the development of an external force ; in such a picture we might find a very fruitful hypothesis of the contractile process.

Certain Applications to Man.

I wonder whether those who approved the rules framed to guide the Croonian Lecturer would have regarded such hypotheses of the nature of muscle as coming under the category of " unsubstantial theories " : lest perhaps they might, let me return, not indeed to an "anatomical demonstration," but to the almost equally solid ground of common observation on man. Why is it so much more tiring to run fast than slow ? How may a man who could run for an hour at one speed exhaust himself completely in 30 or 40 seconds by going twice as fast ? What determines the optimum gear ratio in a bicycle ? the proper size of a spade or a tennis racket ? the length of an oar or the frequency of the stroke in a boat ? It is, in fact, these very viscous-elastic phenomena whose nature we have been discussing.

If a human arm muscle during a maximal isometric contraction be released and allowed to shorten against the inertial resistance of a flywheel, the external work which it does can be determined from the kinetic energy developed in the wheel. By varying the moment of inertia of the wheel, or—what has the same effect—by varying the leverage of the arm upon it, the rate of shortening can be altered at will, and we may find the relation between work and speed. This relation, in man, can be very exactly observed, and we find it to adhere closely to the formula

$$W = W_0 (1 - v/v_1)$$

where W is the work actually performed at speed v, W_0 the work in a similar contraction (allowing for fatigue) at zero speed, and v_1 a constant. It is obvious that a maximum speed exists, $v = v_1$, at which no external work at all is done : and that at speeds slightly less than this the work will be only small. Now suppose that a man doubles his frequency and rate of movement: v/v_1 previously, say, 0·4 becomes 0·8; W previously 0·6 W_0 becomes only 0·2 W_0. Thus to do the same amount of work per movement the effort (measured by W_0) must be multiplied three times. But if the rate of movement be doubled the average kinetic energy of the limbs must be multiplied 4 times: 4 times the work must be performed upon them every step, each unit of which (as we have seen) requires 3 times the effort to produce it; altogether 12 times the effort to run 100 yards at twice the speed!

The optimum gear ratio and speed of movement are determined by similar factors. The work done in a contraction lasting t seconds is $W = W_0 (1 - t_1/t)$ where t_1 is now the shortest possible time of contraction. The slower the contraction the more work it can perform; for given work, the fewer fibres it will be necessary to excite. A prolonged contraction, however, requires energy to maintain it as well as to set it up: this energy increases uniformly with the time, *i.e.*, more rapidly than the work done. Expressing it in a formula, $H = \alpha W_0 (1 + bt)$, where H is total energy used in maintaining an effort W_0 for time t and α and b are constants. The mechanical efficiency of the movement, W/H, is then

$$\frac{1 - t_1/t}{\alpha (1 + bt)},$$

an expression which has a maximum at a certain definite value of t. Thus there exists an optimum time of movement, an optimum speed. If one wants to move a given amount of earth in a given time one may do it with a small spade and a high speed, or with a large spade and a low speed. If one wants to propel a boat a given distance in a given time, one may use a short oar and a rapid stroke, or a longer oar and a slower stroke. There is a limit either way beyond which it is inexpedient to go: for the best possible performance there is an optimum length, an optimum speed.

Such things, without any theory, "unsubstantial" or otherwise, one may deduce directly from the observed facts: the deduction may serve perhaps in place of "a dissection or other anatomical demonstration."

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The Viscous Elastic Properties of Smooth Muscle.

By A. V. Hill, F.R.S., Foulerton Research Professor of the Royal Society.

(Received May 3, 1926.)

(From the Department of Physiology and Biochemistry, University College, London.)

When the striated muscle of a frog is instantaneously released during an isometric tetanus its tension falls immediately by an amount depending on the extent of the release, to zero if the release be more than about 10 per cent. of its length; it then rises again along a curve similar to that of the initial rise of tension when stimulation began. This led Gasser and A. V. Hill (1) to deduce that the processes, whatever they be, which determine the development of a contraction when a tetanus begins, are the same as those which determine the redevelopment of the contraction after a sudden release during a tetanus. The observation and the deduction from it were used by Garner (2) in support of his hypothesis that contraction is due to the formation of some molecular pattern in the muscle substance, of something analogous to liquid crystals on the surfaces or throughout the volume of the fibre.

It was obviously desirable to test this observation—of what is perhaps the simplest and most fundamental of the phenomena described by the term "viscous-elastic"—on some contractile tissue as different as possible from the voluntary muscle of the frog. During a recent visit to the Marine Biological Laboratory at Plymouth, Mr. C. F. A. Pantin and Mr. G. P. Wells introduced me to the longitudinal muscles of the body wall of *Holothuria nigra*, a set of five smooth muscles running the whole length of the animal's body, easy to prepare, able to survive for long periods in sea-water, and capable of being excited, either by single shocks or by a slow tetanus, to produce a very considerable tension.

These muscles develop an isometric contraction, and shorten so slowly that the observations which were difficult on the skeletal muscles of the frog become possible with them with the simplest apparatus. In their natural relaxed state in the full-grown animal they are 15 cm. or more long: they may shorten, however, under quite considerable tension, to less than 25 per cent. of their relaxed length. On removal from the animal they usually shrink right up, but if immersed in sea-water under a small load they gradually lengthen out again. They may be excited by single-break shocks to give a twitch-like contraction (see fig. 1, curves (1) and (2)), or by a constant current (which, however, causes electrolysis) or by a tetanus consisting of making and breaking the primary circuit of a coil by hand about once a second. The induced shocks employed have to be enormously large—they must liberate far more energy than the response of the muscle itself, which from the point of view of myothermic experiments is a serious difficulty—but the regularity with which the muscle behaves over long periods suggests that they do not cause any serious damage to it, and that they evoke a response not largely differing from the one caused by natural stimulation through the nerves.

Experimental.

The muscles are dissected out, a thread is tied to each end, and they are left in sea-water till required. Care must be taken in attaching the thread, as the muscles are very liable, under the high tensions which they develop, to tear away from it. An ordinary Lucas muscle chamber is provided with a vertical pin at the end distant from the recording lever: round this pin the muscle is passed, its two ends being tied together to the internal vertical arm of the crank. The external horizontal arm is then connected by a thread to an isometric tension lever below it. The Lucas chamber is fixed to a Palmer stand, by which it can be raised or lowered by turning a screw. Half a turn of this screw can be made rapidly and quietly by hand in a fraction of a second, thereby releasing or stretching the muscle 2½ mm. The tension lever below is held in a fixed stand, so that its line of zero tension remains constant on the drum, which in these experiments revolved slowly against it at a rate of about 0·6 mm. per second.

Before stimulating the muscle the sea-water is run out, the electrodes being then applied near the two ends. Each muscle as employed was doubled up around the pin, and occupied a length of about 6 or 7 cm. in the chamber. In this way a tension of 80 gr. wt. might be developed during a prolonged tetanus of about one double (make and break) shock per second. A Harvard coil was employed, with the secondary usually completely covering the primary. Such violent stimulation would rapidly destroy a frog's skeletal muscle, but appears to be necessary to secure a maximal response in the smooth muscle of *Holothuria.* The tension lever used had a uniform scale of 1 mm. = 4 grms. wt., with a magnification of about 60.

The muscles, at whatever length they are kept, usually develop and maintain, even when unstimulated for long periods, a certain amount of tension. Many of the records show this. On rare occasions the muscle develops a spontaneous rhythm: in one case it went into rhythmic contraction each time a single-induction shock was applied. In all the experiments discussed below such spontaneous or induced rhythmic contractions were absent.

Results.

In fig. 1 curves (1) and (2) are shown records of the twitch resulting from a single-break induction shock: (1) and (2) are on different muscles, (2) being much slower than (1). Curve (3) represents a tetanus of the same muscle as in curve (2), by make and break shocks at 2 per second. Curve (4) shows an interesting phenomenon: a quick stretch* of the resting muscle produces a sudden rise of tension followed by an obvious response; the first is physical, the second obviously physiological. The first is present in a muscle undergoing a maximal tetanus or contracting in KCl-sea-water (see fig. 2 curves (4) and (9), and fig. 3 curves (2) and (3)), the second is absent under these conditions; since the muscle is already giving a maximal response it cannot respond further to a stretch. This second rise does not always occur; the first is invariable (see, for example, curve (5) of fig. 1). Nor does it result from a quick stretch immediately preceded by a quick release (see curve (6) fig. 1 (R + S)), although it follows the converse order—a quick stretch followed by a quick release (S + R).

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* All stretches and releases shown are of 2½ mm.

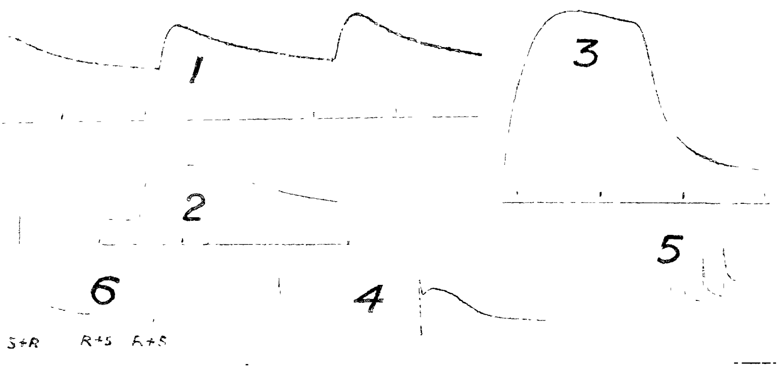

FIG. 1.—Curves (1) and (2), isometric twitches following single-break shocks ; (3) isometric tetanus, same muscle as (2), two make and break shocks per second ; (4) quick stretch of resting muscle, causing sudden rise of tension and subsequent response ; (5) ditto, but no subsequent response ; (6) S + R, quick stretch of resting muscle followed by quick release, showing subsequent response ; R + S, quick stretch preceded by quick release, showing no subsequent response. Time marks (and in figs. 2 and 3) every 20 seconds ; all releases and stretches 2½ mm. ; 1 mm. tension = 4 grms. wt. (*Holothuria.*)

In fig. 2, curves (1), (2) and (3) are examples of the quick release of a muscle undergoing a single twitch. The muscle is that of curve (2), fig. 1, which contracted so slowly that redevelopment of tension was possible even in a single twitch. Curves (2) and (3) of fig. 2 each show two quick releases. From (3) especially the form of the redevelopment is seen to be similar to that of the original development of the response. It is obvious that the tension in a "released twitch" never reaches as high a value as in the original twitch ; relaxation follows too rapidly.

In fig. 2, curves (4) to (9), is shown the strongest confirmation of the observations of Gasser and Hill on frog's skeletal muscle. In all these curves a tetanic stimulus of about one double shock per second began at the left, and the tension rose towards a maximum. When it was somewhere near that value the muscle was released suddenly 2½ mm., the tension dropped, and then began to develop again ; after an interval—the stimulation being continued all the time—it was again released, the process being repeated in some cases as often as 10 times, giving a total release of 25 mm.

In every case the tension redeveloped along a curve similar to, or identical with, the original curve. This is strikingly shown in (5) and (6), where even the finer details of the curve reappear after each release, and also in (4), where,

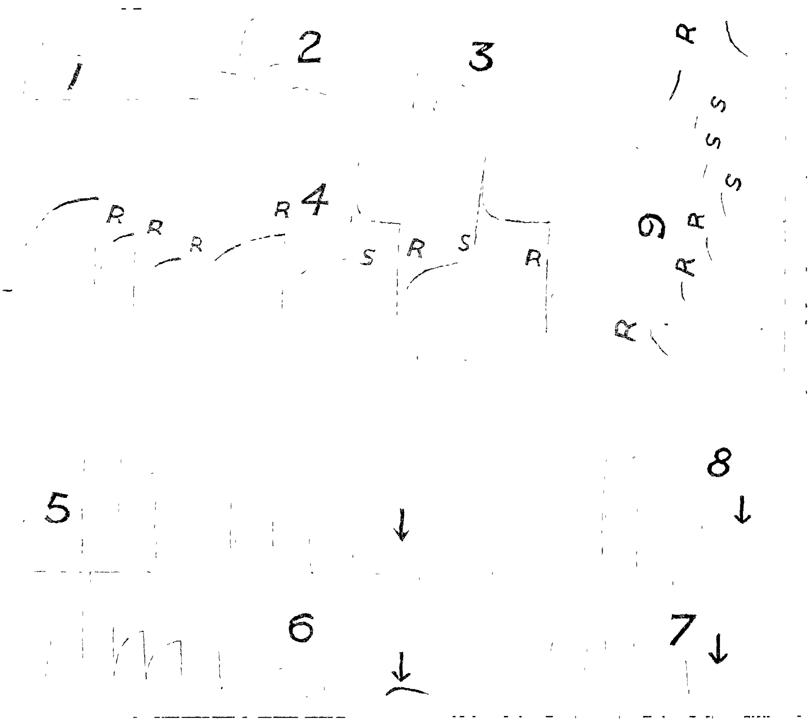

Fig. 2.—Curves (1), (2) and (3), isometric twitch with quick release; same muscle as fig. 1, curve (2); curves (4) to (9), isometric tetanus, about one make and break shock per second, ending at arrow (where shown), with quick releases (R) and quick stretches (S) interposed during the continuance of the tetanus. Curve (4), same muscle as fig. 1, curve (1). (6) Same muscle as (5), 14 hours later. (*Holothuria.*)

after the third release, the drum was allowed to run for about 25 seconds before a further release was given, the resulting curve being almost identical with the original. In some cases the original curve was found to be slightly slower in its rise than that of redevelopment; this was probably due to a sub-maximal stimulus not exciting all the fibres of the muscle at the first shock, and could usually be cured by increasing the stimulus.

Curve (6) is on the same muscle as (5), but 14 hours later. In curves (4) and (9) are shown examples (denoted by S) of a quick stretch during a tetanus. Owing to the very high tensions so realised the muscle was apt to tear, and only a few experiments of this kind were performed; so far, however, as the evidence goes, the fall of tension after the sudden rise produced by the quick stretch is similar in character to the rise of tension following the sudden fall produced by the quick release.

These muscles, like other smooth muscles, are very susceptible to an increase in the potassium in the fluid bathing them. A few experiments therefore were performed on quick releases (R) or quick stretches (S) of muscles contracting under the influence of a high K-content of their surrounding sea-water. Fig. 3 curves (1) and (2), ((2) being a continuation of (1)), are on the same muscle as fig. 2, curve (4), and fig. 1, curve (1). It is obvious that the releases of fig. 3 are very similar to the releases of fig. 2, as also are the stretches. We may conclude, therefore, that *either a stretch, or a release, during activity produced, whether by stimulation or by potassium, results in the same process, a gradual return to the tension characteristic of the new length along a curve similar to, or identical with, the original development of the response.*

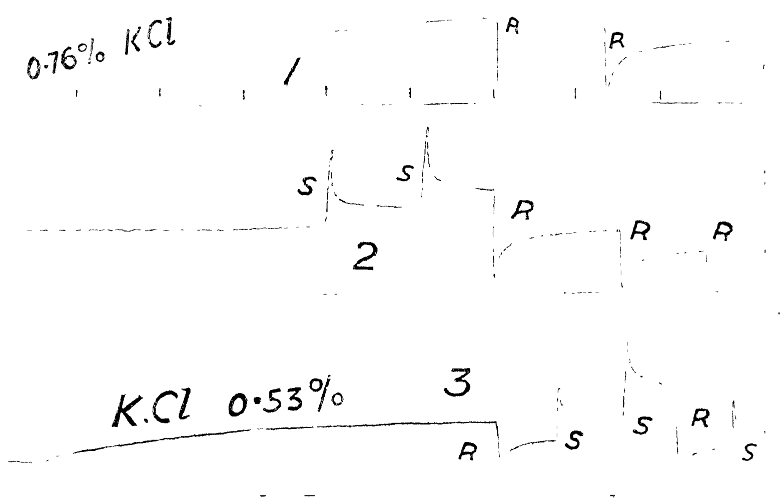

Fig. 3.—Contractions evoked by high potassium content in the surrounding sea-water. Curve (2) is a continuation of (1), with quick releases (R) and quick stretches (S) interposed during the contractions. (1) and (2) on same muscle as fig. 1, curve (1), and fig. 2, curve (4). (*Holothuria.*)

The evidence is strikingly clear, and in view of Garner's suggestions it may be well to inquire more closely what it means. It may be admitted at once that the phenomenon in question is not due to simple physical "viscosity," but to some molecular rearrangement which takes place only slowly, either when the stimulus is first applied, or when the previous arrangement has been shattered, or disorganised, or thrown out of equilibrium by a sudden release or stretch. It would seem that the development of the contraction is due to the formation of some molecular pattern in the muscle fibre, to some process analogous to crystallisation; when the muscle is released its tension can be developed again only by the rearrangement of its elements in a "crystal" of the same molecular pattern, but of different external shape. This is the same process as took place originally on stimulation, and it is natural that the same curve should be followed.

The processes shown in the figures of this paper need a time of the order of half a minute for their completion; the corresponding events in the skeletal muscle of a frog (1) require about 1/5 of a second: it is striking that phenomena so diverse in their time-relations should be found so similar in their general character.

It would be difficult to fit the rapidly moving muscles of a frog into the same hypothesis of simple physical viscosity as the smooth muscle of *Holothuria*: if, however, we may suppose the development of the response in all contractile tissues—not merely muscles—to be due to the formation of a new molecular pattern under the influence of the breakdowns caused by the stimulus, then there is no difficulty in imagining different rates at which the resulting "liquid crystals" form. Quite small changes in molecular constitution—*e.g.*, in the arrangement of the amino-acids in a protein—might have large effects upon

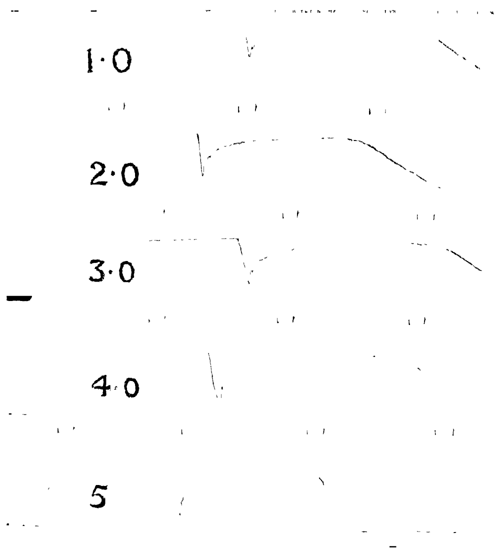

FIG. 4.—Quick release of *biceps cruris* of tortoise during isometric tetanus. Muscle 62 mm. long, room temperature. Seconds. Releases, as shown, 1, 2, 3, 4 and 5 mm.

the rate of formation of such spatial patterns, as they are known to have upon the speed of ordinary crystallisation ; and we should need to look for the cause of the vast differences existing between different contractile tissues, not in any mysterious differences in their mechanisms, but simply in the specific structural chemistry of the material which goes to form their protoplasm.

Note.

The same experiments can obviously be made, in the same way, with any muscle which develops its contraction sufficiently slowly. The method has been applied to the *biceps cruris* of the tortoise, which, on sudden release during a prolonged tetanic contraction, shows a large immediate fall of tension followed by a slow gradual redevelopment. The curve of this redevelopment of tension is identical in shape with that of the initial development when stimulation began (see fig. 4).

Summary.

1. Gasser and Hill found that the sudden release, during an isometric tetanus, of the skeletal muscle of a frog is followed by an instantaneous drop of tension, and then by a gradual redevelopment along a curve identical with that of the original rise when stimulation began. This observation has been repeated, both for quick releases and for quick stretches, on the smooth muscles of *Holothuria nigra*, in which the speed of contraction is less than 1/100 of that of a frog's striated muscle. The return to the tension characteristic of the new length is precisely similar in its time relations to the initial rise of tension.

2. It is concluded that the development of a contraction during stimulation, and its redevelopment after release, are due to the same process, viz., the formation of an organised molecular pattern, arranged in space whether of two or three dimensions in the fibres of the muscles. Such a molecular re-arrangement, either on stimulation or during actual shortening, is the basis of the " viscous-elastic " phenomena found in all contractile tissues.

It is a pleasure to acknowledge my thanks to Dr. E. J. Allen, F.R.S., and Mr. C. F. A. Pantin, of the Marine Biological Laboratory at Plymouth, for the courtesies extended to me during the conduct there of these experiments, and to Mr. Pantin and Mr. G. P. Wells for their suggestion of the use of the *Holothuria* muscles.

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Observations on the Muscles of Normal and Moulting Crustacea.

By J. P. Hoet* and Phyllis M. Tookey Kerridge.†

(Communicated by Prof. A. V. Hill, F.R.S.—Received May 21, 1926.)

In the course of previous work on mammalian muscle (1) it was found that the muscle of rabbits, when glycogen-free, went into immediate rigor mortis when the circulation stopped. In such cases the rigid muscles were found to have an alkaline reaction.

Our attention was called to the following statement of Claude Bernard (2):—

> "Les muscles des crustacés en général, et notamment de l'écrevisse . . . ne contiennent pas en dehors de la mue de matière glycogène. Aussi, après la mort, entrent-ils en rigidité tout en conservant leur réaction alcaline."

The intention of the present work was to investigate this interesting statement, which is repeated on Claude Bernard's authority by Biedermann (3). He also quotes experiments by Kirch (4) on glycogen in crawfish, finding the total glycogen content four months before moulting to be 0·08 per cent. of the body weight, just before moulting 0·40 per cent., and 0·82 per cent. during the moulting period.

Methods.

Estimations of glycogen were by Pflüger's method, as described by Best, Hoet and Marks (5). The hydrogen-ion concentration of the minced muscles was determined by the glass electrode method (6), (7). A portable outfit, including a Lindemann electrometer, was employed.

Observations.

The most remarkable difference between hard-shelled crustaceans and soft-shelled specimens which have just moulted is the greatly diminished activity of the latter. These hardly resist handling and are quite unequal to combat with the stronger, normal individuals. Their muscles appear very fluid in character compared with the normal, strong claw and abdominal muscles.

Glycogen.—Tests for glycogen in the muscles of *Cancer pagurus, Carcinus*

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mænas and *Homarus vulgaris* showed it to be present in considerable amount (comparable with that in mammalian muscle) in hard-shelled specimens, but in small amount in soft-shelled specimens.

Hydrogen-ion concentration.—Estimations of the hydrogen-ion concentration were made of the muscles (abdominal and claw) of normal *Homarus vulgaris, Cancer pagurus, Carcinus mænas, Eupagurus bernhardus* and *Maia squinado,* and of soft-shelled specimens of Homarus, Cancer and Carcinus.

The p_H of all muscles measured as soon as possible after death was about 7·05 for hard and for soft-shelled specimens. The reactions of the muscles in the body are probably slightly more alkaline than this, as acid may have been produced during excision or before the estimation was made. The average time between excision and estimation was about 10 minutes. No liquid air was available into which the muscles could have been plunged immediately after excision, which precaution was taken in other work on the p_H of tissues (1). Twenty hours after death the excised muscles were again tested. Those from hard-shelled specimens were decidedly acid, and those from soft-shelled alkaline. The reactions could easily be observed with moist litmus paper, which was probably the method of Claude Bernard.

Some of the quantitative results obtained are summarised in the following table. The figures given are mean values of a number of determinations, the number being given in brackets.

Glycogen Content.

(Per cent. of fresh weight.)

	Hard Shelled.	*Soft Shelled.*
Carcinus	—	<0·01 (1)
Cancer	0·55 (1)	<0·03 (5)
Homarus	0·19 (2)	—

Hydrogen-Ion Concentration.

(p_H — 20 hours after death.)

	Hard Shelled.	*Soft Shelled.*
Carcinus	6·52 (2)	7·08 (5)
Cancer	6·55 (2)	7·1 (1)
Maia	6·22 (2)	—
Eupagurus	6·22 (1)	—
Homarus	6·46 (3)	7·28 (1)

Rigidity.—After death, the crustacean muscles poor in glycogen went into a state of rigor more quickly than the normal ones, the claw muscle being the first to become rigid. This rigidity is not so well marked as in mammalian muscle, and it is very easily broken. The muscles which have freshly become rigid will react to an electrical stimulus. Later, however, after about 18 hours, the muscles of soft-shelled crabs are in permanent rigidity, while those of hard-shelled crabs are still limp. On immersion in sea water at 36 deg., the muscles of soft-shelled crabs became rigid in about one minute, while those of hard-shelled crabs remained limp for two or three minutes. Other conditions and temperatures were not studied.

Discussion.

We are unable to confirm the statement of Claude Bernard that the muscles of crustacea contain glycogen only at the time of moulting. It is improbable that the many hard-shelled specimens which we tested were all about to moult, as they were brought to the laboratory on various days and from various places, and no new shells were noticed under the old ones. Neither did we find that at the time of moulting the muscles were rich in glycogen. The reverse was, indeed, the case. We do not claim, however, to have made a comprehensive study of the variation of the muscle glycogen content of these crustacea with their life-cycle.

We have been able to show that when the muscles are poor in glycogen they go into a state of rigor while still alkaline. In this point we have confirmed the statement of Claude Bernard. This rigor, conditioned by lack of glycogen and not accompanied by production of acid, comes on more quickly in these muscles than in those containing larger amounts of glycogen. An immediate rigor mortis, however, was not observed at room temperature.

In conclusion, we beg to offer our sincere thanks to Dr. E. J. Allen, F.R.S., and the staff of the Marine Biological Laboratory, Plymouth, for their valuable help and hospitality.

Summary.

1. Glycogen was found in the muscles of normal, hard-shelled crustacea. Only small amounts were found in those of soft-shelled specimens of the same species.

2. An early onset of rigor mortis, associated with alkaline reaction, was observed in crustacean muscles poor in glycogen. When glycogen was present

in the muscles there was a post-mortem development of acid comparable with that in mammalian muscle.

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On the Macro-Chemistry of the Endodermis.

By J. H. Priestley and Edgar Rhodes.

(Communicated by F. F. Blackman, F.R.S.—Received April 13, 1926.)

Introduction.

As our knowledge of the processes of development in the plant body grows in precision, increasing importance attaches to that detailed knowledge of the structure of a tissue system which enables a more correct assessment of its influence upon the growth and development of neighbouring tissues. This is particularly true of that cylinder, one cell in thickness, which is known as the endodermis, which is almost invariably present in the root of a vascular plant, and very frequently found also in the shoot (4). This cylinder passes through a series of developmental stages, and micro-chemical tests indicate that the structural features of these different stages are very distinct. When the cells of the endodermis cease to be meristematic, as they are left behind the growing point, the cell protoplasts of this layer remain embedded in the walls of each cell in the region of the Casparian strip. This strip is continuous around the radial longitudinal and transverse walls of every cell, so that the result is to make this cylinder, one cell in thickness, a cylinder in which the Casparian strip may be visualized as a network, with the meshes in the network completely filled by the living protoplasts.

The protoplasts at this stage are very definitely semi-permeable. Within the endodermis are the vascular elements; these contain water and water-soluble substances, which are able to leak out along the cellulose walls of the cells which surround the vascular channels.

An aqueous sap, sometimes under considerable positive pressure, thus reaches so far as the inner tangential walls of the cells composing the endodermal cylinder. The further passage outwards of the water or solution now depends upon the capacity to penetrate the living protoplast or the Casparian strip. Obviously, then, the nature of the Casparian strip is a question of great interest. The results of its micro-chemical investigation have previously been discussed (4). In this paper a few data as to its nature can be supplied from macro-chemical experiments carried out with an endodermis in the primary stage of Kröemer (2), at which, apart from the Casparian strip, the remainder of the cell walls seem quite normal in character.

Later in the plant's development, the endodermis often passes on to another stage, the secondary stage of Kröemer, of which the characteristic structural feature is the suberin lamella, which is usually, but not always (6), deposited all over the inner surface of the cell wall. This suberin lamella has also been examined micro-chemically. In the flowering plants the conclusion was reached that it consisted mainly of fatty substances impregnating a basal substance akin to cellulose but more resistant to hydrolysing agents (4).

The endodermis of the flowering plant usually passes on to a tertiary stage, in which within the suberin lamella, a layer of cellulose can be detected. Obviously, however, from the standpoint of function, the important feature is the replacement of the protoplast in the meshes of the Casparian strip by a continuous layer of fat-impregnated substance. If the Casparian strip is also fat impregnated, as micro-chemical tests would suggest, this single layer of cells will now prevent the exchange of water and water-soluble solutes between the tissues it separates. The existence of such a secondary or tertiary endodermis may then account for the exfoliation of the cortex in many roots, and many structural features that have been discussed in other papers.

There is a good deal of disagreement in the literature as to the micro-chemical behaviour of the walls of the cells of the endodermis. As the points at issue may be of considerable importance, when a micro-chemical study of this tissue was made some years ago, a beginning was made in the accumulation of material for macro-chemical examination in order to check statements which rested so far only on micro-chemical experiment. As this tissue forms a cylinder only one cell in thickness, it is not possible to accumulate large quantities, although it has been collected continuously during the years 1920–26. With the aid of grants from the Royal Society, the services of students were available during the vacations for the tedious and somewhat delicate task of isolating this cylinder of tissue from rotting plant material. In the meantime, methods for the investigation of tissues of this type were gradually worked out by macrochemical studies of cork (8) and cuticle (3). By 1925 these methods seemed sufficiently tested to permit of their application to the accumulated supplies of endodermis.

The Preparation of Material.

It was pointed out earlier (4), that the stems of *Potamogeton perfoliatus*, L., provide excellent material for the collection of endodermis for macro-chemical investigation, because apart from short tracheids at the node, no lignified xylem is present within the endodermis to resist the action of cellulose-decomposing organisms, when the material is placed in water and allowed to rot.

The only tissues that resist decay and remain coherent are the cuticle and the endodermis, and whilst a sharp movement of the rotting tissue in the water will break the cuticle up into flakes, the endodermis resists such treatment and can thus be drawn out practically free from all other tissues, washed and placed on filter-paper to dry. As described in an earlier paper (4), this endodermal material was originally dropped into concentrated sulphuric acid; cellulose walls were thus dissolved away and nothing left but the slightly carbonised fat-impregnated membranes of the endodermis. The treatment with sulphuric acid, however, might materially alter the chemical nature of the layers it was desired to examine, and as further experience showed that upon prolonged digestion by micro-organisms in water the endodermis could be freed from all internal tissues, from 1921 onwards the treatment with sulphuric acid was given up and the endodermal cylinder collected after maceration in water. In the young elongating internodes this cylinder is in the primary stage, but in the adult internodes it is mainly in the tertiary stage, in which a cellulose lamella may be found within the suberin lamella. The bulk of the material collected would thus be in the tertiary stage, and any fatty substances extracted from the walls would come both from suberin lamella and Casparian strip. In 1925, therefore, young elongating internodes and adult internodes were treated separately, so that data could be obtained enabling a comparison to be made between the fatty substances derived from the Casparian strip alone, and those obtained from Casparian strip and suberin lamella together.

From 1923 onwards, material has also been collected in a similar way from the roots of hyacinth (*Hyacinthus orientalis*, L.). The roots were cut off the bulbs when about six inches long, the apices cut off about an inch below the tip, and then these lengths of root placed in water and allowed to rot. Under this treatment the tissues all disintegrate, save exodermis and endodermis; and, as these are quite separate from one another, the endodermis could be gently disengaged from within the exodermis by means of a dissecting needle, and collected for macro-chemical analysis. The endodermis thus obtained was mainly in the primary stage.

As the result of several years' collection, there became available by the winter of 1925-1926 about 110 grams of dried endodermal strips of Potamogeton stem and 0·6 grams of endodermis of the root of the hyacinth.

The Chemistry of the Impregnating Substances.

The removal of these substances was effected by the method used by Rhodes (8) in work upon the suberin lamella of potato cork, which involves five successive operations :—

(*a*) Removal of free fat and fatty acids by extraction with chloroform.

(*b*) Saponification of the remaining tissue with 3 per cent. alcoholic soda, to break up complexes and liberate the constituents as sodium soaps.

(*c*) Removal of unsaponifiable material by extraction of the aqueous suspension of the above saponification products with purified ether.

(*d*) Formation of free acids by acidification of the liquid freed from unsaponifiables, and extraction of the fatty acids suspension with petroleum ether B.P. 40°-60° C., in order to remove normal fatty acids, and leave in suspension fatty acids which are hydroxylated or oxidized, and which are called " oxy " acids.

(*e*) Removal of " oxy " acids by filtration.

All the above operations are performed in semi-quantitative manner.

Table I gives the quantitative results of the above series of extractions with the four types of material available.

Types of Material.—(*a*) Mainly Tertiary Endodermis (Potamogeton) collected and stored over a period of three years in corked bottles.

(*b*) Tertiary Endodermis (Potamogeton) freshly prepared and treated within seven days of preparation.

(*c*) Primary Endodermis of Potamogeton freshly prepared and treated at once.

(*d*) Endodermis of hyacinth, freshly prepared and treated at once.

Samples (*c*) and (*d*) were very small in amount, and special precautions were taken during manipulation to ensure the usual degree of accuracy.

Table I.

		Percentage. Free Fat.	Percentage. Unsaponifiable Material.	Percentage. Normal Acids.	Percentage. Oxy. Acids.	Percentage. Oxy. and Normal Acids / Free Fat.
Potamogeton	(*a*) Tertiary Endodermis, stored	I 1·05	0·60	0·63	1·04	1·59
		II. 1·06	0·60	0·63	1·06	
	(*b*) Tertiary Endodermis, fresh.	1·34	1·13	0·265	1·14	1·08
	(*c*) Primary Endodermis, fresh.	5·3	2·54	1·14	3·20	0·82
Hyacinth	(*d*) Primary Endodermis, fresh.	16·3	1·08	0·81	12·7	0·83

The subsequent treatment of the separated products was by reason of their small amount restricted to a determination of the Iodine Numbers of the fats obtained from two chloroform extracts. The fat from the stored tertiary endodermis gave an Iodine Number of 46·1, that from the fresh tertiary an Iodine Number of 60·3.

From these results it will be seen that each sample of tissue contains some free fat, which will account for the micro-chemical behaviour towards fatty stains. Further, each contains a considerable amount of fatty material in some combined form, from which the constituents can only be released on saponification. Such complexes characterize the suberin deposit of potato cork (Rhodes (8)), and the cuticle (Lee (3)), and conclusions from micro-chemical studies as to the presence of similar complexes in both the primary and secondary endodermis are thus confirmed.

Turning to the figures for the ratio $\frac{\text{Oxy. Acids and Normal Acids}}{\text{Chloroform soluble Fat}}$ (which represents practically the ratio of free to combined fatty material) and comparing these with similar figures for potato cork, which are of the order of 3·3 to 3·6 for normally formed layers, and 4·0 to 4·8 for cork tissue formed over wounds, it seems that in the case of endodermis the figures are invariably lower than those of cork, indicating a smaller proportion of fatty acid complexes in relation to free fatty bodies.

It has been demonstrated that the occurrence of these complexes depends on the air supply (5), and since the corky layer of the potato is so much more freely exposed to air than the endodermis, which is in communication with air only through the adjacent external intercellular spaces, the lower figure for combined complexes for the latter tissue might have been predicted. Further, in so far as it is permissible to attach meaning to the variation in this ratio for the different endodermal types, the fact that the tertiary endodermis shows a higher figure than the primary, and the air-stored strips a still higher value, lends support to the view that the suberin lamella in the secondary and tertiary stages contains fatty substances which are more completely oxidized than those in the young primary endodermis, as a natural consequence of the longer exposure to air. Similarly, in the case of potato cork the proportion of free fatty material in normally formed layers is always higher than that in the regenerated layers which are more exposed to air. Further, in the case of potato cork, samples of the *same material* showed a reduction in actual free fat on exposure to air, from which it seems probable that it is the free acids and fats which contribute to the formation of the complex combined materials in the suberin layer, and the same would appear from the figures to be true of endodermis. It has further been observed micro-chemically that the Casparian strip of the primary endodermis is more acid in character than the suberin lamella of the secondary (4). The free acids, which largely determine acidity, will be found in the chloroform extract. The chloroform soluble material is highest in the primary endodermis, and lowest in the stored tissue, so that micro- and macro-chemical data would again appear to be in agreement.

The facts, therefore, lend good support to the micro-chemical evidence favouring the view that the Casparian strip and secondary endodermis contain complex fatty acid derivatives, and that, in the secondary stage at least, these are of a nature similar to those found in a suberized membrane such as that of the potato.

It is also interesting to note that there was no definite evidence for phellonic acid (7) in the present and previous investigations on endodermis, nor was any found in potato cork (8).

The Basal Substances of the Casparian Strip.—The micro-chemical investigations revealed the fact that the Casparian strip and the suberin lamella each possess and are built up on a basal substance, and that the basal material of the strip is different from that of the suberin lamella. The latter, there is reason to believe, is a carbohydrate, whilst that of the strip is of a much more resistant type and of a nature quite unknown. It was obviously important to endeavour to find out a little more about the composition of this body, and while it must be admitted that nothing definite can yet be said, the work has produced one or two interesting facts and negatived some other possibilities.

The first and most obvious line of attack was to remove everything but the basal substance and determine what elements were present in it. Accordingly some tissue, freed from fat and suberin, was thoroughly extracted with Schweitzer's reagent to remove cellulose, and incidentally any alkali-soluble protein. Having made certain by microscopic examination, that the basal substance had not been removed, the fact that the basal substance is insoluble in concentrated sulphuric acid was utilized to free it from any remaining organic bodies, including the resistant base to the suberin lamella.

The transfer to and recovery from sulphuric acid was carried out, so far as possible, quantitatively. From twelve grams of air-dry Potamogeton endodermis, which represents more than half the amount collected in one year, 11·3 grams were removed by Schweitzer's reagent. The residue of 0·7 grams yielded, after treatment with concentrated sulphuric acid and subsequent dilution with water, about 0·17 grams of what could only be regarded as an impure basal substance.

It has previously been reported that the basal substance contained nitrogen and sulphur (4, *loc. cit.*, p. 123). This rested upon macro-chemical analysis of the residue obtained by dropping the original macerated strips directly into concentrated sulphuric acid. In the present case, however, the material is first treated with Schweitzer's reagent, and when the residue is then dropped into concentrated sulphuric acid, a product is obtained which after thorough washing proves to be quite free from either nitrogen or sulphur. On the other hand the previous extraction with Schweitzer's reagent has not only removed cellulose, but also a substance containing nitrogen and sulphur, tests for these elements giving positive results when applied to the precipitate thrown down on addition of hydrochloric acid to the Schweitzer's reagent after its removal from the endodermis. This precipitate gave in bulk the reaction for cellulose with suitable iodine reagents, but occasional masses in the precipitate remained simply yellow with iodine.

No definite protein tests were given by the material, but there can be little doubt that it represents the remains of the protoplast surface which was embedded in the Casparian strip. When examined microscopically after treatment with such a reagent as hydrochloric acid, the cells of a primary endodermis invariably show the protoplast still attached to the Casparian strip, although everywhere else they are contracted completely away from the wall. Under such circumstances it is easily understood that when the basal substance of the Casparian strip is treated with a strong alkaline reagent, like Schweitzer's reagent, not only is all, or nearly all, the cellulose removed, but along with it come away the nitrogen and sulphur-containing substances which thus bound the protoplast to the strip. The possibility of the nitrogen and sulphur arising from the disorganized remains of the protoplasts *in the cell* seems excluded, as these will have been completely removed during the vigorous alkaline hydrolysis that the endodermis has undergone during the removal of the fatty substance.

These compounds containing nitrogen and sulphur persist, because they are held so firmly in the wall, and in view of the fact that after treatment with concentrated sulphuric acid they may still be found in the Casparian strip, the conclusion seems justified that they are closely built into its fabric, although the original conclusion (4), that they are chemically combined with this basal substance, no longer seems justified. Similarly it is found in the hyacinth root that the basal substance of the Casparian strip remains after some 80 per cent. of the fat residue has been removed by Schweitzer's reagent. This substance also resists concentrated sulphuric acid, but was too small in amount to admit of further examination. The impure basal substance of Potamogeton on ashing was found to contain approximately 5 per cent. of inorganic matter, which failed to show anisotropism under the polarizing microscope, and did not give the sodium silico-fluoride micro-chemical test for silica. Examined microscopically, it still showed the network system characteristic of the basal substance, but at the same time it is completely soluble in dilute hydrochloric acid. As the whole of the inorganic matter represented only about 5 per cent. of the whole of the basal substance, the only conclusion possible is that the inorganic matter has thoroughly impregnated the Casparian strip, which of course is not surprising in view of its position and function. On ignition it retains the characteristic form of the network in just the same way that a string after soaking in inorganic salts can yet retain the form of the string after complete combustion of the original fibre. That the constituents of this inorganic matter were of the usual type left in plant ash was demonstrated by deliberately ashing some untreated endodermis. The ash contained iron (ferric), calcium, magnesium, potassium, aluminium, and sodium.

It has also been noted that if endodermis were treated with concentrated sulphuric acid, and the acid subsequently diluted with water, the residual material—the basal substance contaminated with something containing nitrogen—was susceptible of oxidation with concentrated nitric acid. Proceeding in this manner a solution was obtained which presumably contained oxidation products of the basal substance, and which would probably be acid in character. It was found first that this strongly acid solution on dilution with water deposited a dark brown precipitate, insoluble in ether, chloroform, petroleum ether, ligroin, benzene and alcohol. No solvent for this body has yet been discovered, and its nature is quite unknown. After removal of this precipitate the solution yielded to ether extraction a substance which when deposited from ethereal solution formed flakes with a peculiar honeycomb structure as seen under the microscope. Owing to the fact that only about 0·3 grams were available further investigation was out of the question; but this substance may possibly be similar to that which Von Höhnel records in micro-chemical experiments and describes as "cerin" or "cerinic acid" (1). Of what still remained in solution it can only be said that it contained but a trace of one of the water-soluble acids which would be expected after such treatment, while other quite unlikely acids for which tests were made were also absent. Oxalic acid was present in traces, while acetic, meconic, succinic, hippuric, cinnamic, salicylic, tannic, gallic, and pyrogallic acids were absent. This section of the work has, therefore, produced little save a suggestion for further work on the same lines when enough material can be obtained to yield fractions sufficiently large for further examination.

The data obtained, therefore, suggest that the basal material of the strip, like that of the suberin lamella, contains no nitrogen, and is impregnated with inorganic bodies such as are normally found in plant ash, and that by treatment with concentrated nitric acid the basal substance yields a brown body insoluble in water and dilute acids, and also an ether-soluble body, waxy in appearance, neither of which is as yet identified.

Summary.

As the result of macro-chemical methods of investigation the following statements may be made about the endodermis:—

(1) The primary endodermis contains free fat and other fatty derivatives, in some form of combination, yielding on saponification normal and oxidized fatty acids.

(2) In its secondary and tertiary stages the endodermis contains suberin-like materials similar to those found in potato cork.

(3) As in potato cork, the suberin lamella arises from fatty substances as the result of oxidation and drying processes. The relative increase in the combined insoluble fatty anhydrides, as in the case of regenerated potato cork, appears to take place at the expense of free fat or fatty acid.

(4) The basal substance of the Casparian strip contains no nitrogen, but some

substance containing nitrogen and sulphur is closely knit into the fabric of the strip.

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A Comparative Study of Turacin and Hæmatin and its Bearing on Cytochrome.

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I. Introduction.

This study originated from the investigation on cytochrome, which is a widely distributed, intracellular, respiratory pigment. It was found in the course of this investigation that many problems concerning this pigment would be more easily approached, or even solved, when we have succeeded in elucidating certain obscure points concerning some of the better-known and simpler compounds such as turacin and hæmatin. It was found, for instance, of paramount importance to obtain a better understanding of the relationship between the physico-chemical state of the pigment and its absorption spectrum. It will be shown here that the definite change in the absorption spectrum of a substance, which passes from a state of a solution into that of a colloidal suspension, explains a number of phenomena which have been previously observed but not understood. The combination between the reduced or oxidised hæmatin and various nitrogen compounds, in other words the relationship between such compounds as hæmatin, hæm, hæmochromogen and kathæmoglobin will also be considered. This will be shown to have an important bearing on the study of cytochrome.

The methods used in this investigation do not differ much from those already described in my study on cytochrome (1925, pp. 313–314). Two instruments have been used for this study: the microspectroscopic ocular of Zeiss and the Hartridge reversion spectroscope, the latter having been used both in the ordinary way and as a microspectroscope. The sources of light have varied according to requirement, and were either ordinary filament electric bulbs (50 candle-power), pointolite lamp, Nernst lamp or a small arc. The latter was very useful in the spectroscopic examinations of turbid fluids and precipitates. We can hardly overestimate the importance of the last-named method, for a number of essential spectroscopic properties of the pigments can be revealed only by the study of their solutions during the process of precipitation.

II. TURACIN.

(*A*) *Main properties and absorption spectra.*

Turacin is a red pigment, which was described by Church (1870, 1892) from the feathers of the wings of 16 species of Touracos, African birds belonging to the family of Musophagidæ. He found that this pigment contains 7 per cent. of copper, and in strong sulphuric acid yields a porphyrin very similar to the ordinary hæmatoporphyrin. He found also that the pigment is insoluble in acids but soluble in weak alkali, giving a red solution with a very characteristic absorption spectrum. In the feathers the bands are nearer the red end of the spectrum than in solution. The composition of turacin may be represented according to Church by the formula: $C_{82}H_{81}N_9Cu_2O_{32}$. Laidlaw (1904) prepared synthetically a copper-porphyrin compound which in alkaline solution shows some resemblance to turacin. Fischer and Hilger (1923) have shown that the porphyrin of turacin is very similar, if not identical, with uroporphyrin, and that turacin appears to be an excretory product by means of which a toxic light sensibilising product such as uroporphyrin is eliminated from the body.

The recent advance in our knowledge of hæmoglobin and its derivatives made it desirable to reinvestigate the properties of turacin (which is a copper-porphyrin compound) and to compare them with those of the iron-porphyrin compounds. The following are the main results of these investigations, which partly corroborate Church's early observations, but also reveal a series of new and interesting properties of this pigment (fig. 1):

(1) The absorption-spectrum of turacin (Tr) in feathers shows two wide strong bands: (I) an asymmetrical α-band with its longer wave side darker (at 587 $\mu\mu$) and centre at 583 $\mu\mu$ and (II) a β-band with its centre at 542 $\mu\mu$. The width of the bands shown by Tr in the thickness of one feather correspond approximately to: α-599·5–567·5; β-558–525·5. When 3 to 4 superposed feathers are examined with a microspectroscope and a stong source of light, another very faint band γ can be seen nearer the red end of the spectrum at 621 $\mu\mu$. It is possible, however, that this band belongs to a distinct compound, such as a break-down product derived from turacin. The type of absorption spectrum of turacin as seen in the feather we shall refer to as type *A*.

(2) Tr is soluble in weak solutions of alkali (NH_3, NaOH, K_2CO_3, etc.), giving a purplish-red solution, with the absorption bands lying nearer the blue end of the spectrum than in the feather: α-562·5, β-526. Two other very faint shadings noticed by MacMunn (Church, 1892) can be seen only in a more concentrated solution of the pigment: γ at 597 $\mu\mu$ (which seems to correspond to band γ of feather-turacin), and δ at 484 $\mu\mu$ which somewhat resembles the third band found by Dhéré and Vegezzi (1917) in various hæmochromogen compounds. The type of absorption spectrum of Tr as seen in weak alkaline solutions we shall call type *B*.

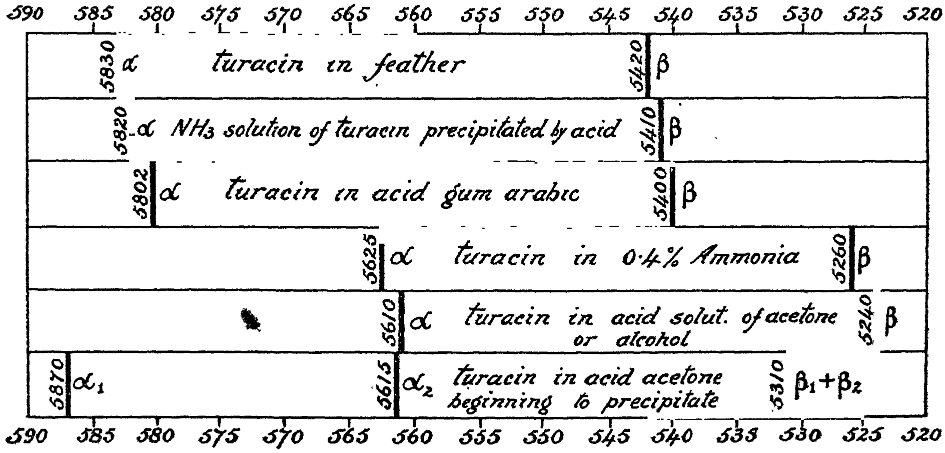
590 585 580 575 570 565 560 555 550 545 540 535 530 525 520
5830 α turacin in feather 5420 β
5820 α NH3 solution of turacin precipitated by acid 5410 β
5802 α turacin in acid gum arabic 5400 β
5625 α turacin in 0·4% Ammonia 5260 β
5610 α turacin in acid solut. of acetone or alcohol 5240 β
5870 α1 5615 α2 turacin in acid acetone beginning to precipitate 5310 β1+β2
590 585 580 575 570 565 560 555 550 545 540 535 530 525 520

FIG. 1.—Positions of the absorption bands in turacin under various conditions.

(3) Feather Tr is insoluble in acids, weak or strong, and on acidifying the alkaline solution of Tr the pigment is precipitated. Its colour is changed from purplish-red to orange-red, and its absorption spectrum reverts to the type *A* found in the feather : α-582, β-541.

(4) The addition of gum arabic or gelatin (0·2 per cent. to 0·4 per cent.) to the alkaline solution of Tr which has afterwards been acidulated prevents the precipitation of the pigment. The latter changes its colour, as in Experiment 3, and its absorption bands also shift towards the red end of the spectrum : α-580·2, β-540. The transparent fluid thus obtained can be kept indefinitely. The gum arabic or gelatin (which do not combine with turacin) act here only as protective colloids, keeping the pigment in the state of colloidal suspension.

(5) When alcohol or acetone is added to alkaline Tr and the solution is then acidulated, Tr does not precipitate, but remains in solution, with an absorption spectrum of type *B* : α-561, β-524.

(6) The acid turacin precipitate, having the absorption spectrum of type *A*, when shaken and slightly warmed in strong alcohol or acetone, shows a remarkable change in its absorption spectrum, most noticeable in the α-band. The shorter wave side of this band at first becomes darker and then the band splits into two : α_1 lying close to the position of the maximum intensity of the α-band in feathers and α_2 which occupies the position of the α-band in alkaline solution. On further heating or boiling this alcoholic solution, band α_1 disappears, while α_2 becomes more intense and the absorption spectrum becomes identical with that of Experiment 5. On cooling the solution the band α_1 reappears and the pigment begins to precipitate (fig. 1).

(7) Feather Tr, or dry Tr precipitate, does not dissolve in cold or boiling absolute alcohol, whether alkaline or acid ; but when a very small amount of water is added to hot alcohol, Tr passes into solution even in the presence of strong acids and gives the absorption spectrum of type *B* : α-562·7, β-526.

(8) Tr is insoluble in chloroform or in a mixture of acetic acid with ether.

(9) Treated with strong alkali, Tr gives compounds with absorption spectra of types intermediate between those of *A* and *B*. For instance : (*a*) in strong NaOH solution, the positions of the bands are : α-569·5, β-530 ; (*b*) dried in NaOH, the bands shift further towards the red end of the spectrum : α-575 ; (*c*) in strong K_2CO_3 solution the bands are : α-569, β-530* ; (*d*) when this solution is dried to a powder the absorption spectrum of the pigment becomes of

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* It may be interesting to note that when a piece of ordinary filter-paper is stained with this solution, the absorption spectrum reverts to that of ordinary weak alkaline solution—in other words, the bands shift towards the blue end of the spectrum.

type *A* : α-579·5, β-536; (*e*) when alkaline solution of Tr is added to absolute alcohol containing a solid piece of NaOH, turacin precipitates and its absorption spectrum becomes similar to that of the acid precipitate (Experiment 3).

(10) Various salts, such as NaCl, NH_4Cl or K_2CO_3, added to the weak alkaline solution of the pigment shift the band of the latter in the same way as strong NaOH : α-569, β-530. On addition of alcohol or acetone to these solutions the absorption spectrum reverts to the type *B* as seen in a weak alkaline solution.

(11) The salts mentioned above do not precipitate the pigment, but shift its absorption bands from 45 to 70 Å towards the red end of the spectrum. The addition of the salts of divalent kations, such as Bä or Cä, to the alkaline solution of Tr precipitate the pigment and shift its absorption bands more than 120 Å towards the long wave end of the spectrum.

(*B*) *Different types of absorption spectra and the state of the pigment.*

These experiments show that turacin has two distinct main types of absorption spectra: type *A*, with bands lying nearer the red end of the spectrum, and type *B*, with the bands lying nearer the blue end of the spectrum. In order to explain the origin of such variation we must now discuss the three following possibilities: (1) that the positions of the bands vary only with the chemical composition of the pigment, (2) that they follow Kundt's rule, and finally (3) that they vary with the state of dispersion of the pigment (Svedeberg and Ostwald).

(*a*) *Absorption spectra and composition of turacin.*—It is quite natural to suppose, first of all, that the types of absorption spectra of turacin correspond to two distinct compounds, acid and alkaline. This supposition, however, cannot be accepted, as it does not explain the experiments showing that the absorption spectra of acid and alkaline Tr in alcohol or acetone are of the same type *B*, while alkaline or acid precipitates of Tr have the absorption spectra of the type *A*.

(*b*) *Kundt's Rule.*—This relationship was expressed by its author in the following manner: "If one colourless solvent has a distinctly greater refractive and dispersive power than a second solvent, then on dissolving an absorbing substance in the two the absorption bands will be nearer to the red end in the case of the first solvent than they do in the case of the second" (translation in Baly's *Spectroscopy*, vol. 1). Although a number of the cases observed seem to follow this rule, a great number of exceptions have been found which do not obey it. Thus, according to Formánek (see Kayser, 1905, III, 82), of 524 coloured substances dissolved in various solvents and examined spectroscopically, only 284 followed this rule, while in the remaining cases the absorption bands either did not move with the change of the solvents, or moved in the direction opposite to that expected under Kundt's rule. In the case of turacin, taking only in consideration the substances in which the pigment passes into a true solution, the positions of the absorption bands vary very little with the change of the solvent.* Thus the α-band of turacin in the different solutions used varies as follows :—

Alkaline water	562·5	Acid methyl alcohol	561·5
,, ethyl alcohol	564·0	,, ethyl alcohol	561·5
,, propyl alcohol	565·5	,, amyl alcohol	565·2
,, glycerine	564·2	,, ethyl alcohol mixed with xylol	565·2

The maximum shift of the α-band shown in these examples is approximately 37 Å.U. This shows that the so-called Kundt's rule does not help in the least to explain the variations in the positions of the bands of different types of spectra in turacin, which, as we have seen, may reach 200 Å.

(c) *Absorption spectra and degree of dispersion of a pigment.*—This relationship indicated by Stenger (1888) and elaborated by Svedberg (1909) and Wo. Ostwald (1911, 1918), is that the maximum absorption of light changes with the degree of dispersion of the substance, and moves towards the violet end of the spectrum as the degree of dispersion increases. This relationship adequately explains the various absorption spectra of turacin. Thus, the type *A* of absorption spectrum of turacin, with its bands lying near the red end of the spectrum, is always given by Tr in its lowest degree of dispersion, namely: in solid state in the feathers, in acid precipitate, in alkaline precipitate and in acid colloidal suspension. The type *B* of absorption spectrum, with the bands lying near the short wave end of the spectrum, is given, on the contrary, by turacin in its highest degree of dispersion, namely, when it passes into a real solution such as: (*a*) aqueous, slightly alkaline solution, (*b*) alcoholic or acetone solution, whether alkaline or acid. We have seen also that in acid alcohol or acetone both types of spectra (*A* and *B*) can be seen simultaneously, corresponding respectively to the pigment in a fine precipitate (*A*) and in a solution (*B*). As in the case of solutions, with the rise of temperature when more Tr passes into solution, the absorption bands of the type *A* become faint and may disappear, while those of the type *B* become stronger. At lower temperatures the dissolved turacin partly precipitates, the intensity of the absorption spectrum of the type *B* decreases, while that of the type *A* increases.

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* The refractive indices of some of the pure solvents used in these experiments, as given in the Table of Physical and Chemical Constants (Kaye and Laby, 1921) are :—water—1·33; methyl alcohol—1·33; ethyl alcohol—1·362; amyl alcohol—1·41; glycerine—1·47; xylol—1·497.

In these experiments the transition between the dissolved and precipitated pigment is so rapid that we cannot follow spectroscopically the successive degrees of molecular aggregation. On the contrary, in the presence of stronger alkali, as shown in Experiment 9, turacin exhibits stages intermediate between the absorption spectra of type *A* and *B*. That the shift of the bands towards the red end of the spectrum in a strong NaOH solution is due to the aggregation of the turacin is shown by the fact that a similar shift may be produced when, instead of alkali, ordinary salts (NaCl, $CaCl_2$, etc.) are added to the weak alkaline solution (Experiment 10).

It is evident therefore that in turacin the variations in the position of the bands are mainly due to the changes in the molecular aggregation of the pigment. In this respect turacin shows a very great analogy with certain dyes, such as congorubin, investigated by Ostwald (1918), Haller (1920) and others (*cf.* also section *C* under hæmatin).

(*C*) *Some properties of turacin compared with those of hæmatin.*

Turacin from feathers resembles hæmin only in being soluble in alkali, and, like pure hæmatin, can be brought from alkaline into acid solution (aqueous-colloidal or alcoholic). It differs, however, from hæmatin and other iron-porphyrin compounds in several striking properties, the principal ones being :—

(1) Turacin does not combine with any of the nitrogen compounds, such as ammonia, pyridine, nicotine, or albumin, which enter so easily into combination with hæmatin.

(2) Tr, with its hæmochromogen-like spectrum, is not affected by air, O_2 or CO, passed through its alkaline or acid solution.

(3) Tr is not changed by the addition of strong oxidising agents such as H_2O_2, potassium ferri-cyanide or potassium permanganate.

(4) Tr is not changed by the addition of strong reducers such as $Na_2S_2O_4$.

(5) Tr gives neither oxidase or peroxidase reactions with guaiacum or benzidine; we know, on the other hand, that metallic Cu and its salts, such as

$CuCl_2$, $CuSO_4$ and copper acetate, give good oxidase and peroxidase tests,* and that all hæmoglobin derivatives which retain iron give good peroxidase reactions.

It is interesting to note that while *copper*-protein compounds, such as hæmocyanin and iron-*porphyrin* compounds, form a series of important respiratory pigment or catalysts, the *copper*, when combined with *porphyrin*, as in turacin, gives a very stable but completely inert compound.

Of all the metallic derivatives of hæmatoporphyrin examined by Hill (1925, and unpublished observations) only those with iron, cobalt and manganese show an oxidation and reduction effect, and of these compounds only that with iron unites with nitrogen compounds.

III. HÆMATIN AND HÆMOCHROMOGEN COMPOUNDS.

(*A*) *Hæm, alkaline and acid hæmatins.*

In a series of valuable papers Anson and Mirsky (1925) have definitely proved that hæmochromogen prepared from Hb is a conjugated protein, consisting of an iron-porphyrin compound and globin, and that other hæmochromogens can be obtained by replacing globin with various N-compounds. They have given the name of "hæm" to the iron-porphyrin compound obtained by dissolving hæmin crystals in NaOH. According to these authors, reduced hæm, combined with a N-compound, yields hæmochromogen, while oxidised hæm, combined with a N-compound, gives hæmatin, the latter being the oxide of hæmochromogen. While the difference between the reduced "hæm" and hæmochromogen was proved by them beyond doubt, the difference between the "oxidised hæm" and hæmatin rests mainly upon the difference in the solubility of these two compounds. Acid hæmatin prepared from hæmoglobin is soluble in water, while "oxidised hæm" prepared from hæmin, when treated with acids, precipitates. By applying here the same method as for turacin (Experiment 4) we can show, however, that this difference is only apparent. On adding to the alkaline solution of hæmin (in NaOH) 0·2 per cent. to 0·4 per cent. of gum arabic and then acidulating the solution, "hæm" does not precipitate, but remains in a colloidal suspension protected from precipitation by the gum arabic.† The fluid remains clear and transparent, while its absorption spectrum is the same as that of acid hæmatin prepared from hæmoglobin or the suspension of "hæm" precipitate. We know, on the other hand, that "hæm" does not combine with gum arabic, for on reduction in alkaline solution it does not give hæmochromogen, but ordinary reduced "hæm." It is evident, therefore, that acid hæmatin prepared from Hb is a colloidal suspension of hæmatin, protected by globin from precipitation, but not united with it.

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* It is interesting to note that hæmocyanin, in spite of being a respiratory pigment, is devoid of oxidase or peroxidase properties.

† Similar result is obtained in using, instead of gum arabic, a solution of potassium silicate.

Moreover, an aqueous alkaline solution of hæmatin, prepared from hæmoglobin, has an identical absorption spectrum to a similar compound prepared from hæmin, provided the same concentration of alkali (NaOH) be used in both cases. Alkaline or acid alcoholic solutions of hæmatin prepared from Hb also have similar absorption spectra to the corresponding compounds prepared from hæmin. Finally, the absorption spectrum of alkaline oxidised hæmatin, prepared from hæmin, is not changed when a nitrogen compound, such as albumin, pyridine, or nicotine, is added to the solution.

Ordinary hæmatin or oxyhæmatin is therefore the same substance as the "oxidised hæm" of Anson and Mirsky, being an iron-porphyrin compound free from protein. The term "hæm" should be reserved for the reduced hæmatin only when the latter is devoid of an N-compound. In other words when hæmochromogen in alkaline solution is oxidised, it becomes completely dissociated into its iron-porphyrin and protein components; when hæmatin is reduced it unites with a protein, if one is present, and hæmochromogen is re-formed.*

(*B*) *Parahæmatin* (= *Kathæmoglobin*).

We have dealt so far with hæmatin as an alkaline or acid compound, each being free from globin or other nitrogen compounds and each having a characteristic absorption spectrum, well known and described by previous authors. *Neutral hæmatin* can also be prepared in the form of a colloidal suspension by neutralising alkaline hæmatin dissolved in gum arabic (0·2–0·4 per cent.).

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* This is not in agreement with the view expressed by Gamgee ('Schäfer's Text-Book of Physiol.,' vol. 1, p. 251). According to this author the absorption spectra of hæmatin and oxidised hæmochromogen differ only in the extreme violet and in ultra-violet regions. Whilst the former shows a general absorption of light in these regions, the latter is, on the contrary, transparent to these rays. It is quite possible, on the other hand, that this difference is merely due to the action of reagents used in the preparation of the compounds. We know, for instance, that the concentration of alkali used in preparation of hæmatin has a very great influence upon its general absorption of light and the position of its absorption band in the visible part of the spectrum. We may mention also that, according to Gamgee, Soret's band is absent in both hæmatin and his oxidised hæmochromogen.

The absorption spectrum of this compound seems to show the coexistence of faint, hardly perceptible, bands of both acid and alkaline hæmatins.

This neutral hæmatin, obtained from hæmin, must not be confused with the "neutral hæmatin" described by Arnold (1900). This, as was shown by van Klaveren (1901), is a conjugated protein, named by him "kathæmoglobin" (KatHb). Various methods have been used for preparing this compound. According to Arnold, the easiest method consists in shaking methæmoglobin solution with strong alcohol. Ordinary Hb or HbO_2, shaken with strong alcohol, chloroform or acetone, also yield this compound. Van Klaveren obtained it from freshly prepared and washed crystals of Hb in the following manner: 100 cc. of strong solution of these crystals were mixed with 200 cc. of 96 per cent. alcohol containing 1–2 cc. of concentrated KOH. The solution was then warmed to 60° C., neutralised with HCl, cooled, precipitated and washed with water and finally redissolved in alcohol 60 per cent. containing some NaCl. The simplest method for preparing KatHb in aqueous solution was recently discovered by Adair (1926); it consists in dialysing ordinary alkaline hæmatin (prepared from Hb) against pure distilled water and changing the latter until it becomes neutral or even slightly acid* (pH-5·8). The red fluid thus obtained has the same properties and the same absorption spectrum as the "neutral hæmatin" of Arnold or "kathæmoglobin" of van Klaveren. The absorption spectrum shows two bands: a narrow and weak α-band lying at 567·5 μμ and a much wider and stronger β-band with its centre at 531·2 μμ. On addition of ammonium sulphide, ordinary globin hæmochromogen is obtained, and this, on shaking, reverts to KatHb. The addition of acids or alkalies to the solution gives respectively acid or alkaline hæmatins, which, as we know, are free from protein or other nitrogen compounds. On boiling KatHb solution, it changes its colour from red to brown, and the absorption bands disappear or are replaced with those of ordinary neutral hæmatin. On cooling the solution, the absorption spectrum of KatHb reappears.

This interesting observation of Arnold, confirmed by Klaveren and by the writer, shows that with the rise of temperature KatHb becomes dissociated into hæmatin and globin, and that these on cooling reunite again.

Compounds similar to KatHb are obtained when neutral hæmatin is combined with other nitrogen compounds. Thus, the hæmatin-pyridine compound which, according to Anson and Mirsky (1925, p. 59), has an absorption spectrum resembling that of HbO_2 is, in fact, of the same nature as KatHb. It shows two·characteristic absorption bands: a narrow and faint α-band lying at 564 and a stronger and wider β-band with its centre at 527·5. Mr. R. Hill informs me that neutral hæmatin unites also with nicotine and histidine, giving compounds similar to KatHb (see fig. 2). Moreover, according to Dhéré and Vegezzi (1917), the absorption spectrum of oxyhelicorubin has a striking resemblance to that of Arnold's neutral hæmatin. In oxyhelicorubin, which is a natural pigment, the α-band, with its centre at 571·7, is also narrower and fainter than the β-band, which lies at 533·6. Finally, as will be shown in the last chapter, the oxidised hæmochromogens *b′* and *c′* of cytochrome also have each all the appearance of a hæmatin-nitrogen compound. It is evident therefore that we are dealing here with a definite group of compounds, of which KatHb is only one member. As the other members of this group are devoid of globin, the latter being replaced by various other nitrogen compounds, it is hardly possible to apply to all of them the term kathæmoglobin. I propose therefore the name *parahæmatin*, which may be used as a more general term applying to all the hæmatin-nitrogen compounds, including kathæmoglobin.

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* When hæmatin prepared from hæmin is dissolved in alkaline gum arabic and dialysed against distilled water, ordinary neutral hæmatin is obtained. To obtain KatHb the presence of globin is essential.

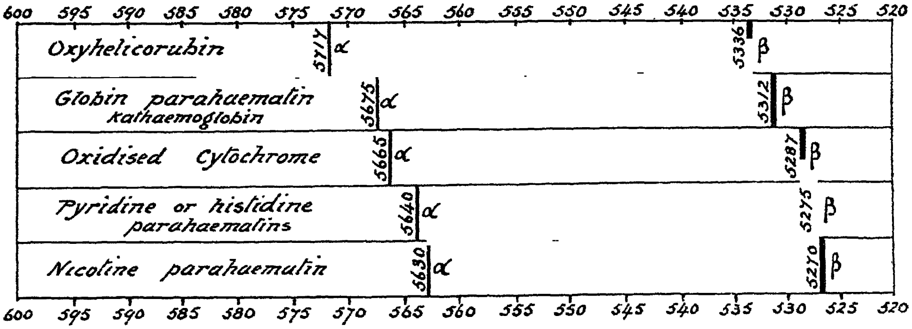

FIG. 2.—Positions of maximum intensities of the absorption bands in various parahæmatin compounds.

Parahæmatin (= Arnold's neutral hæmatin = kathæmoglobin of van Klaveren) is therefore an oxyhæmatin united with a nitrogen compound such as: globin, histidine, pyridine, nicotine, and the unknown nitrogen compounds of cytochrome and of oxyhelicorubin. Parahæmatin exists only within a fairly limited range of *p*H and has a characteristic absorption spectrum of two rather diffuse bands, of which α is narrower and fainter than β. On reduction parahæmatin gives a corresponding hæmochromogen; on addition of alkalies or acids it gives respectively alkaline or acid hæmatin.

The relationship between the various compounds mentioned in this section can be easily summarised schematically, as is shown in fig. 3.

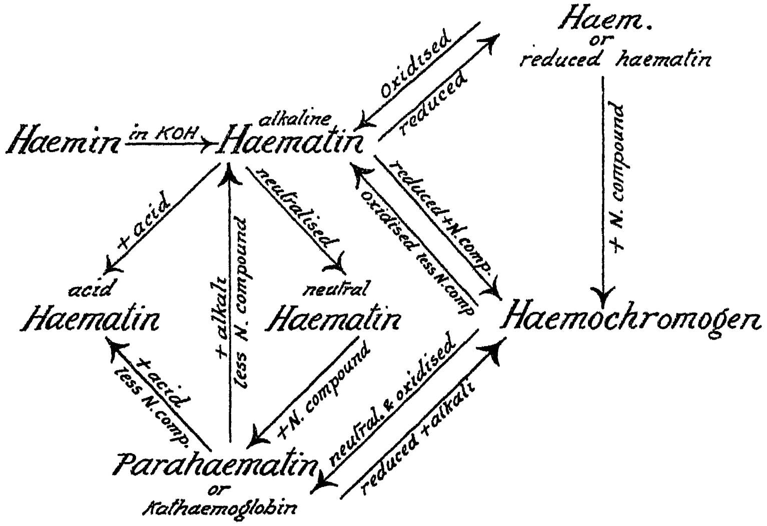

FIG. 3.—Diagram showing the relationship between hæmin, hæmatin, hæm, parahæmatin and hæmochromogen compounds: the expression "less N-compound" indicates that the compound becomes dissociated into an iron-porphyrin and the N-compound, such as globin, for instance.

(C) *Absorption spectra of hæmatin and hæmochromogen and the degree of their dispersion.*

The study of hæmatin and hæmochromogen also provides a series of important examples demonstrating the relationship between the absorption spectrum of a substance and its degree of dispersion :—

(1) The absorption spectra of acid hæmatin in a true alcoholic solution or in an aqueous colloidal suspension are of the same type, but the bands of the former are 110–115 Å.U. nearer the short-wave end of the spectrum than those of the latter.

(2) Alkaline hæmatin, prepared by dissolving hæmin crystals in potassium carbonate,[*] when reduced, begins to precipitate, giving the characteristic

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[*] When hæmatin is dissolved in NaOH or KOH and then treated with alcohol or acetone a peculiar compound is formed, with a characteristic absorption spectrum, described by Dhéré and Vegezzi (1916) as one of their hæmochromogen compounds. This peculiar hæmatin NaOH compound shows a sharp and narrow α-band at 595 and a wide β-band at 558. These two authors obtained the same compound by mixing blood hæmochromogen with strong alcohol.

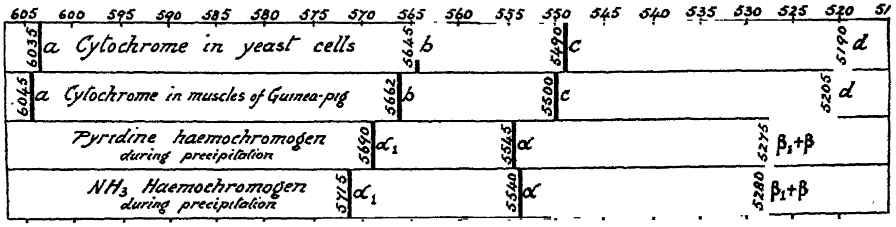
absorption spectrum of hæm or reduced hæmatin. The same compound is soluble in alcohol or acetone, and shows a similar absorption spectrum, but having the bands 130 to 150 Å.U. nearer the short-wave end of the spectrum. It is important to note that in both experiments (1) and (2) the bands shift in a direction opposite to that expected by Kundt's rule.

(3) Pyridine-hæmochromogen is soluble in strong pyridine and gives a clear and transparent solution with the α-band at 556. When more water is added to the solution it becomes less transparent, and the α-band shifts towards the red end of the spectrum as far as 559·2.

On addition of NaOH the band becomes asymmetrical and lies at 561·5 μμ. By varying the relative proportion of water, pyridine and NaOH one can obtain a turbid red fluid which, on shaking, shows a characteristic suspension of fine precipitate. Examined with the microspectroscope in a strong light, it shows an absorption spectrum composed of three bands : α — 554·5, α_1 — 569 and $\beta + \beta_1$ — 527·5.

On varying the relative amount of alkali and pyridine the absorption spectrum undergoes a marked change. Thus, when more pyridine is added to the solution, it becomes more transparent, band α_1 becomes fainter, while α increases in intensity, and β-band, which is composed of two bands fused together ($\beta + \beta_1$), shifts towards the blue end of the spectrum. On addition of more alkali the fluid becomes more turbid, band α fades away, while α_1 becomes more intense, and $\beta + \beta_1$ shifts towards the red end of the spectrum. Pyridine hæmochromogens may, therefore, show two kinds of absorption spectra—one (*A*) with its absorption bands lying near the red end of the spectrum and given by the pigment when its molecules are aggregated, and the other (*B*) with the bands lying near the blue end of the spectrum and given by the pigment in a true solution (fig. 4).

(4) Ammonia-hæmochromogen is soluble in fairly strong ammonia and shows its α-band at 554. This compound precipitates, however, on addition


605
600
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555
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545
540
535
530
525
520
51
6035
a Cytochrome in yeast cells
5645
b
5490
c
5190
d
6045
a Cytochrome in muscles of Guinea-pig
5662
b
5500
c
5205
d
Pyridine haemochromogen during precipitation
5690
α1
5545
α
5275
β1+β
NH3 Haemochromogen during precipitation
5715
α1
5540
α
5280
β1+β

FIG. 4.—Position of the absorption bands of 3-banded pyridine- and ammonia-hæmochromogens compared with those of cytochrome in yeast cells and in muscles of guinea-pigs.

of salts such as NaCl, NH_4Cl or K_2CO_3, and the turbid fluid thus obtained, when examined with the microspectroscope, shows a 3-banded absorption spectrum: α–554, α_1–571·5 and $\beta + \beta_1$–528. On varying the relative amounts of ammonia and salts one can obtain an absorption spectrum showing either both bands, α and α_1, equally strong, one of the bands much stronger than the other, or finally a spectrum with only one α-band. In other words, NH_3-hæmochromogen also shows two types of absorption spectra: A and B, corresponding respectively to the pigment in the state of a precipitate or of a real solution (fig. 4).*

(5) The double or geminated structure of the α-band in reduced acid helicorubin observed by Dhéré and Vegezzi (1917, p. 58) is very probably of the same nature and due to the presence of helicorubin in two distinct degrees of dispersion.

Several important conclusions can be drawn from these observations:

(*a*) That the absorption bands of the pigment move towards the red end of the spectrum as the degree of dispersion decreases, so confirming the main results of our study of turacin.

(*b*) That a hæmochromogen without changing its N-compound can exhibit two distinct absorption spectra with a difference in the position of the α-bands reaching almost 170 Å.

(*c*) That these two distinct absorption spectra may be seen simultaneously in a fluid showing three absorption bands: two separate α-bands (α and α_1) and a third one composed of two fused β-bands. This 3-banded absorption spectrum has a striking resemblance to a portion of the cytochrome spectrum and will be discussed later under the heading of the latter (fig. 4).

(*d*) That the degree of dispersion of the substances must be kept in mind while they are compared spectroscopically.

These results also give important indications as to the origin of the α and β hæmochromogens recently described by Anson and Mirsky (1925, p. 54). According to these authors, when just a little NH_3 or pyridine is added to reduced hæm, α-hæmochromogen is formed. By further addition of such a compound the bands shift towards the blue end of the spectrum, until they finally reach a definite position, which is not modified by any further addition of such an N-compound, the β-hæmochromogen being thus formed at the expense of the α-form. They have also noticed that the α-hæmochromogen is less soluble than the β-form and easily precipitates out of solution.

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* NH_3-hæmochromogen, showing the 3-banded spectrum, is most easily prepared by dissolving hæmin in strong NH_3 and then adding a little of $Na_2S_2O_4$ distilled water and some NaCl until the fluid becomes turbid. The precipitation of this hæmochromogen was kindly suggested to me by Mr. R. Hill.

It seems certain, however, from our experiments described above, that the α and β-hæmochromogens of Anson and Mirsky do not differ by the amount of the N-compounds with which they are combined, but that they represent only two distinct degrees of dispersion of the same compound. The shift of the bands of the α-hæmochromogen towards the blue end of the spectrum on addition of more NH_3 or pyridine is easily explained as being due to the solvent action of these compounds.*

It is interesting to note, however, that the absorption bands of HbO_2, whether in solution, as precipitate, or dried on a filter paper, occupy invariably the same position. In other words, contrary to the examples examined previously, the absorption spectrum of HbO_2 seems to be independent of the degree of its dispersion. This contradiction may be, however, only an apparent one. It is possible that the shifting of the absorption bands during the precipitation occurs only when the portions of the pigment molecules responsible for the absorption spectrum come sufficiently close in contact with one another. In HbO_2, on the other hand, the pigment-portion of each molecule (iron-porphyrin), even during precipitation, is probably widely separated from its neighbour by the globin which forms a far larger portion of the molecule. It is therefore possible that in HbO_2 the absorption bands remain always in the same position, because the pigment-portions of the molecules never become aggregated.

In this respect we may mention here an interesting observation by Stenger (1888), who found that when a dye such as Magdala red (naphto-safranin) is dried in ordinary conditions, its absorption spectrum and other optical properties undergo a marked change. When, however, the solution is dried in an organic jelly, all its optical properties remain the same as those of a solution. According to Stenger, this is due to the fact that the aggregation of the molecules, as it takes place in the solid dye, is here prevented by the fixation of the molecules in the surrounding hardened medium.

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* As to the shift of 40 Å, mentioned by these authors for pyridine hæmochromogen, it does not represent the actual distance between the centres of α and α_1-bands of these two hæmochromogens, which, as we have seen, may reach 140 Å. It corresponds only to an intermediate phase of aggregation before the stage of precipitation is reached (see Experiments 9, 10 and 11 with turacin).

IV. CYTOCHROME.

(*A*) *Reduced cytochrome and hæmochromogen compounds.*

Cytochrome, as was shown in my previous paper (1925), is an intracellular respiratory pigment widely distributed in the animal and vegetable kingdoms. It can be readily seen in a great variety of organisms, such as bacteria, bakers' yeast, cells of the bulb of eschalot, various cells of worms, snails, the thoracic wing muscles of honey-bees, muscles and other tissues of pigeons and guinea-pigs. In all these organisms (which represent only a few examples among the numerous forms examined), cytochrome in a reduced state has a very characteristic absorption spectrum, composed of 4 main bands, *a*, *b*, *c*, *d*, occupying approximately the same position in each. Thus, in the muscles of a guinea-pig: *a*–6045, *b*–5662, *c*–5500, *d*–5205, the last band being composed of three secondary bands fused together. It was also suggested that cytochrome is not a single compound, but a complex composed of three hæmochromogens, *a′*, *b′*, *c′*, each showing its two characteristic α- and β-bands (fig. 5).

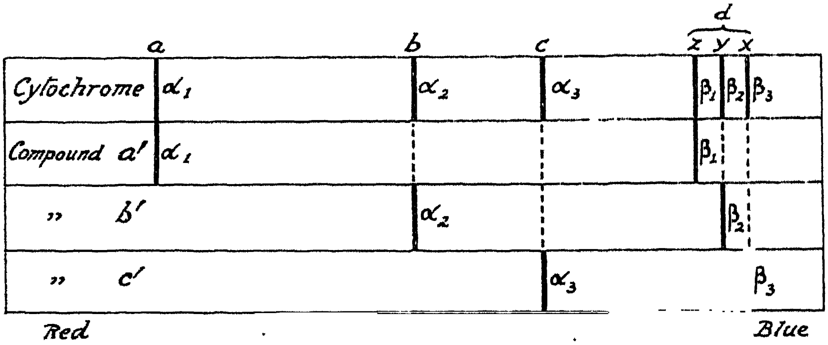

FIG. 5.—Diagram showing the hæmochromogen components *a′*, *b′* and *c′* of cytochrome.

According to Anson and Mirsky (1925) the iron-pyrrol nucleus of two of these hæmochromogens, *b′* and *c′*, is the same as that of hæmoglobin. They ascribed also the difference between the two hæmochromogens as being due to the difference in the composition of the nitrogen compounds which are united with the nucleus. They then propounded a very ingenious theory of equilibrium between the "hæm" (iron-pyrrol nucleus) and the various nitrogen compounds competing for combination with it. According to this theory, the hæmochromogen compounds *b′* and *c′*, which can be represented as N_1Hc and N_2Hc, under special conditions, may have their nitrogen compound N_1 and N_2 replaced by other nitrogen compounds, N_3 and N_4, having a greater affinity for hæm or a greater concentration. Upon this theory is based their explanation: (1) of the variation in the absorption spectrum of cytochrome when the latter is brought under the influence of different factors, and (2) of the relationship between ordinary hæmochromogen and cytochrome compounds in plant cells.

I may mention here that one of the difficulties in the way of this explanation is that the difference in the position of the α-bands of *b′* and *c′* hæmochromogens in cytochrome is at least 160 Å, while hæmochromogens prepared from "hæm" and various nitrogen compounds do not usually give so great a difference in the position of their bands. For instance, having compared the globin hæmochromogen with others prepared from "hæm" and albumin, edestine, peptone, histidine, glycocol, nucleic acid, nicotine, pyridine, piperidine, hydrazin hydrate, acetonitril, quinine, it was found that the position of the bands vary comparatively little. In a number of cases it varies hardly at all, while in others its variation amounts to 20 up to 30 Å. When the lowest number of this series is compared with that given for the α-band of helicorubin, the difference may reach 60 Å. Only two compounds, KCN and cafein, gave hæmochromogen with the bands nearer the red end of the spectrum. We must remember, however, that the nitrogen compounds which form hæmochromogens *b′* and *c′* of cytochrome must be substances of wide distribution and may be expected to form constituents of the cells of all the organisms, bacteria, yeast, higher plants, and animals, since cytochrome has been found in all these organisms.

The results of the experiments with turacin, hæmatin and hæmochromogen, related in previous chapters, show that another explanation can be offered which accounts in a much simpler way for all the difficulties concerning the *b′* and *c′* hæmochromogen of cytochrome. Let us suppose that both these hæmochromogens are composed of the same hæm and nitrogen compound, but differing only in the degree of their dispersion. While *c′*, with its α-band nearer the short-wave end of the spectrum (550 μμ), represents the hæmochromogen in its highest degree of dispersion, *b′*, with its α-band nearer the red end of the spectrum (566 μμ), represents the same hæmochromogen in its highest degree of aggregation. In other words, compound *c′* corresponds to the hæmochromogen in solution, while the compound *b′* belongs to the same hæmochromogen in a state of a colloidal suspension. The equilibrium between these two states of the hæmochromogen may vary from one organism to another, so giving absorption spectra with different relative intensities of the bands.

We are dealing here therefore with a case showing much analogy with pyridine or NH_3-hæmochromogen. Now, considering that the double absorption spectrum, with its characteristic geminated α-band, can be so easily obtained in a test-tube in the case of an ordinary hæmochromogen, it may all the more be expected to occur in the case of hæmochromogen present in a heterogeneous colloidal system such as the protoplasm of a cell. Moreover, this explanation is supported by the following considerations:—

(1) The absorption bands *b* and *c* of cytochrome are very narrow and the band *c* is sharply defined; these characters are seen only in the geminated bands of NH_3 and pyridine-hæmochromogens, when these substances are present in two distinct degrees of dispersion.

(2) The distance between the centres of bands *b* and *c* of cytochrome (about 160 Å) is intermediate between the distance separating the centres of geminated bands (α and α_1) of NH_3-hæmochromogen (170 Å) and that of pyridine-hæmochromogen (140 Å) (fig. 4).

(3) The absorption bands *b* and *c* of cytochrome in plants (Keilin, 1925, pp. 395–396), may be formed at the expense of a single band (at 556 $\mu\mu$) and under the action of an ordinary salt (NaCl)—in other words, in the same manner as are formed the geminated bands of NH_3-hæmochromogen.

We have seen so far that an artificial hæmochromogen can be obtained showing three absorption bands resembling those of cytochrome. To make the resemblance more complete, we shall next have to determine the conditions under which the geminated α-bands of these hæmochromogens shift towards the blue end of the spectrum, while a fourth band develops in the region of the band *a* of cytochrome. These conditions are easily realised, and consist in oxidation of a hæmochromogen with potassium ferricyanide, followed by a reduction with sodium hydrosulphite. Very interesting results have been obtained on applying this method of treatment to nicotine-, pyridine-, globin-, KCN- and ammonia-hæmochromogens. In all these cases we have obtained at first a three-banded absorption spectrum, with one band lying fairly close to the position of the band *a* of cytochrome, and with the two other ordinary hæmochromogen bands (α and β) slightly shifted towards the blue end of the spectrum.* On further oxidation and reductiont he α-band of globin- nicotine-

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* Somewhat similar results have been previously obtained by Sorby (1876) for helicorubin and by Dhéré and Vegezzi (1917) for helicorubin and hydrazin hydrate hæmochromogen. They have found that when helicorubin is oxidised with potassium permanganate and then reduced the two absorption bands shift slightly towards the blue end of the spectrum, while a third band appears and can be seen for some time at 587 $\mu\mu$.

and pyridine-hæmochromogens becomes double, being replaced by two geminated bands, the relative intensities of which vary with the solution. The absorption spectrum finally consists of four bands, corresponding respectively to the bands *a*, *b*, *c*, and *d* of cytochrome.

The position of the bands *b* and *c* is fairly constant for each hæmochromogen, but their relative intensities vary from one solution to another. The band *d* shifts slightly towards the red end of the spectrum when the intensity of the band *b* increases, and towards the blue end when the band *b* fades away. This is in accord with our previous results, and shows that the band *d* is composed of at least two β-bands corresponding to two hæmochromogens, of which *b* and *c* are the two α-bands. The position of the band *a* may also vary in each hæmochromogen from 6030 to 6080 Å, and this seems to be connected, mainly, with the degree of dispersion of the pigment; thus when the band *b* is more intense the band *a* moves towards the red end of the spectrum.

The following are the positions of the bands in nicotine-hæmochromogen when the band *b*, although much fainter than *c*, is distinctly visible: *a*–6060, *b*–5645, *c*–5480, *d*–5200. In pyridine-hæmochromogen under the same conditions: *a*–6050, *b*–5665, *c*–5485, *d*–5200. In globin-hæmochromogen the band *a* is more difficult to obtain, and it never reaches such an intensity as in two previous cases. The position of the bands are: *a*–6055, *b*–5690, *c*–5508, and *d*–5225. The appearance of the band *a* and the shifting of other bands towards the blue end of the spectrum are undoubtedly due to some rearrangement in the pigment molecule itself, produced by the oxidation of the compound. The splitting of the α-band indicates, however, the presence of the pigment in two distinct degrees of dispersion, which is produced by various salts present in the solution. On shaking the solution with air the four absorption bands of these hæmochromogens fade away and reappear only on addition of a reducer, such as sodium hydrosulphite. On shaking the solution with CO, ordinary CO-hæmochromogen is formed. Fig. 6, which represents two absorption spectra of cytochrome (as seen in yeast cells and muscles of Guinea-pig), side by side with those of the modified artificial hæmochromogen, shows that the bands of the latter occupy nearly the same positions as those of cytochrome. We see therefore that an artificial hæmochromogen with only one nitrogen compound may give rise to a mixture of compounds displaying a characteristic four-banded absorption spectrum very similar to that of cytochrome.

These results suggest that cytochrome may also belong to a hæmochromogen compound present in two distinct degrees of dispersion and partly modified

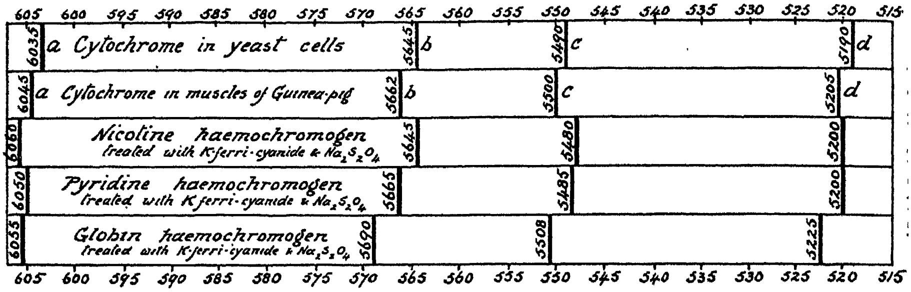

Fig. 6.—Position of the bands of cytochrome compared to those of pyridine-, nicotine- and globin-hæmochromogens treated with potassium ferricyanide and reduced with $Na_2S_2O_4$.

by the active processes of oxidation and reduction which are constantly taking place in living cells and in which these compounds are directly involved.

Before we accept this supposition, however, we must explain one obscure point concerning the changes in the absorption spectrum of cytochrome when cells containing it are treated with certain reagents. When yeast cells or bee muscles are extracted with pyridine a three-banded absorption spectrum is seen in the extract (Anson and Mirsky, 1925, p. 166). This absorption spectrum belongs to two compounds, one with the bands of a typical pyridine-hæmochromogen which seems to derive from compounds *b′* and *c′* of cytochrome, a second with the α-band at 5830 and corresponding to the component *a′* of cytochrome. This experiment shows, according to Anson and Mirsky, that while the iron-pyrrol nucleus of components *b′* and *c′* is the same as in hæmoglobin, that of the component *a′* is different.

We see therefore that while the absorption spectrum of cytochrome in cells is markedly changed on addition of pyridine, ammonia, KOH, etc., the 4-banded absorption spectra of the pyridine-, nicotine- or globin-hæmochromogens are not much influenced by these reagents. This difference is, however, only an apparent one, and can be explained in the following way. The cells of most of the organisms which I have examined, in addition to cytochrome, or even in the absence of it, contain a certain amount of unmodified hæmatin which is not bound to any nitrogen compound and which is therefore invisible. When pyridine or ammonia, added to the cell suspension, reach this hæmatin, they combine with it and in the presence of a reducer form an ordinary hæmochromogen, the α-band of which mixes with the band *c* of cytochrome. In other words, pyridine- or ammonia-extract of all cells contains a mixture of hæmochromogens derived from cytochrome and from ordinary unmodified hæmatin. This mixture explains the complicated appearance of the absorption spectrum of cells treated with pyridine, ammonia or other reagents, and it also indicates that the iron-porphyrin or hæmatin nucleus of cytochrome may not be identical with that of hæmoglobin.

(*B*) *Oxidised cytochrome and parahæmatin.*

The study of parahæmatin compounds gives important evidence as to the nature of oxidised cytochrome. In my previous paper (1925, p. 319) it was mentioned that oxidised cytochrome shows two very faint bands which could only be measured approximately. By means of an improved arrangement of light I have since succeeded in examining the absorption spectrum of this compound more carefully in the thoracic muscles of bees. It shows two bands: a narrow α-band, with its centre at 566·5 μμ, and a stronger and wider β-band, with the centre at 528·7 μμ. These bands (shown in fig. 2) occupy an intermediate position between those of kathæmoglobin and pyridine parahæmatin. The position and relative intensities of these bands in cytochrome clearly indicate that oxidised cytochrome must also belong to the group of parahæmatin compounds, or hæmatin united with a nitrogen compound. Moreover, knowing the properties of these compounds, and especially their existence within a fairly limited range of *p*H, it is natural to expect that in a living cell, within a physiological range of *p*H, the oxidised hæmatin would only be present in the form of a parahæmatin compound.

V. SUMMARY.

(1) Turacin, which is a copper porphyrin compound, differs from iron-porphyrin compound in that (*a*) it does not combine with nitrogen compounds, (*b*) it does not show an oxidation or reduction effect, and (*c*) it does not give a peroxidase reaction.

(2) The absorption spectrum of turacin varies with the degree of dispersion of this pigment; the bands shift towards the long-wave end of the spectrum as the degree of dispersion decreases.

(3) Alkaline hæmatin prepared from hæmoglobin and from hæmin crystals is the same substance, both being an iron-porphyrin compound devoid of protein. When alkaline globin-hæmochromogen is oxidised it becomes dissociated into globin and hæmatin compounds. On reduction, the hæmatin combines with globin to form hæmochromogen.

(4) Acid hæmatin prepared from hæmoglobin is a colloidal suspension of

hæmatin, which is not united with globin, but is protected by the latter from precipitation.

(5) Hæmatin around the neutral point and within a limited range of pH combines with nitrogen compounds and gives parahæmatin compounds, of which kathæmoglobin is one.

(6) The positions of the absorption bands in hæmatin and hæmochromogen compounds (ammonia and pyridine) also depend upon the degree of dispersion of these compounds, the bands shifting towards the short-wave end of the spectrum as the dispersion increases.

(7) In ammonia- and pyridine-hæmochromogens the shift of the α-band towards the red end of the spectrum, as the degree of aggregation increases, may reach respectively 170 and 140 Å.

(8) When such a hæmochromogen is present in two states simultaneously, in solution and fine suspension, it shows a characteristic 3-banded absorption spectrum, in which the two α-bands have a geminated appearance.

(9) Hæmochromogens composed of hæm and various nitrogen compounds, such as globin, pyridine, nicotine, etc., on oxidation with potassium ferricyanide and reduction with sodium hydrosulphite display 4-banded absorption spectra, resembling that of cytochrome.

(10) Evidence is brought forward that the components of reduced cytochrome derive from one hæmochromogen compound present in two distinct degrees of dispersion (b' and c') and partly modified (a'), by the active process of oxidation and reduction in which these components are directly involved.

(11) Oxidised cytochrome has all the characters of a parahæmatin compound.

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Observations on the Œstrous Cycle of the Albino Mouse.

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I. *Introduction.*

Cyclic changes in the female generative tract, correlated with ovulation, and constituting the uterine as compared with the ovarian cycle, have been described for the great majority of mammals. In the past, however, the difficulty of detecting the changes in the intact animal has retarded both the study of the phenomena themselves, as they normally occur, and the use of the cyclic changes as part of the technique for experimental work on the physiology of reproduction. In rodents, particularly, the outward and visible signs of the various stages of the œstrous cycle are almost negligible, and, in the absence of the male, it is notoriously difficult to detect the period of œstrus in the intact animal by superficial examination.

One part of the reproductive tract—namely, the vagina—is, however, accessible for examination, and cyclic changes of the vaginal epithelium corresponding to changes in the uterus were described by Morau as early as 1889. No particular advantage, however, was taken of this fact until the important discovery by Stockard and Papanicolaou (7), in the guinea-pig, that desquamated epithelium could be removed periodically from the vagina, and that the nature of this epithelium varied with the stage of the œstrous cycle arrived at. The elaboration of this discovery into a technique for the detection of the œstrous cycle may be said to have originated the accurate study of the œstrous cycle in spontaneously ovulating rodents, and to have made possible the use of the uterine cycle as part of experimental technique.

That the same procedure can be applied to other mammals has been shown by Long and Evans (5) in their classic work on the rat, and by Allen (1) for the mouse; in each case the onset of œstrus is accompanied by cornification of the vaginal epithelium. In the rabbit, also, vaginal changes indicative of œstrus have been described by Tsu (8), though the fact, first shown by Heape (4), that ovulation in the rabbit is not spontaneous and only occurs after copulation, makes these observations of less practical importance.

Allen has dealt with the vaginal contents in the normal unmated mouse together with the correlation between the changes occurring in the vagina and the changes in the other reproductive organs, and on this basis has studied the normal cycle in the unmated mouse. The work described in the present paper was begun in order to control an investigation which was being made into the effects of X-ray sterilisation on the œstrous cycle, but rather more ground has been covered, and the influence of various conditions on the duration of the di-œstrous period has been studied. It should be emphasised that the work described in this paper is based entirely on Allen's work. The correlation between the uterus, vagina and ovary demonstrated by Allen has been largely assumed for this work. Some few corroborative autopsies have been made, but for the most part the vaginal contents have been taken as being sufficient guide to the œstrous cycle. In view of Allen's work, this is quite a reasonable working assumption.

The mice used in these experiments were drawn from a colony maintained, during the time these experiments were being made, partly by a grant from the

Ministry of Agriculture and Fisheries, and partly by a grant from the Royal Society.

II. *Methods and Material.*

The management of the mouse colony has been fully described in other papers dealing with the same group of animals, and only the details of the special technique used in the experiments to be described will be dealt with here.

Detection of the œstrous cycle.—During the whole of the adult life the vagina of the mouse contains a greater or less amount of material, which may be removed on a spatula, transferred to a slide and examined. Originally the vulva was first dilated with a speculum, but later it was found that the mere insertion of a spatula, given a rotary twist, was sufficient to collect any desquamation present. Allen used Delafield's hæmatoxylin and eosin for staining the smears, but methylene blue was found to be very efficient and easier to manage with large numbers. After staining in methylene blue for about 15 minutes the slides were washed, dried and examined.

The description given by Allen of the nature and composition of the vaginal contents during the various stages of the œstrous cycle is as follows :—

Di-œstrus :—Epithelium (chiefly nucleated) and leucocytes are present during this stage. The vaginal smear tends to be stringy.

Pro-œstrus :—Lightly staining nucleated epithelial cells. During this stage the vaginal contents are serious in nature.

Œstrus :—Non-nucleated, eosinophilous, cornified cells constitute the smears during this period. As this stage begins, the vaginal epithelium may be dry ; subsequently the contents of the vagina become granular.

Meta-œstrus :—The œstrous period is followed by a large infiltration of leucocytes which gradually remove the cornified elements. During this stage the vaginal contents become first pasty and then milky.

Since the di-œstrous period usually lasts two days, and the other three periods about one day each, it follows that although each period will probably be detected, examination once a day may not detect the finer graduations. Thus the pro-œstrus and the first part of the œstrous period (when the epithelium may be dry) may not both be detected in the same cycle by daily examinations. Allen's original observations were made at very frequent intervals, but now that the serial order of the changes has been worked out, once-daily smearing is quite sufficient to keep a close check on the cycle. For each animal examined a smear chart was made, on which day of observation, nature of cells found, amount of smear, and any special features (vaginal plug, etc.) were noted day by day.

Copulation.—Those animals in which it was desired to study the effect of copulation on the cycle without the complication of pregnancy were mated with vasectomised bucks, and later (since copulation is the ultimate criterion of the occurrence of the œstrous cycle) it became a routine to mate with operatively sterile males. As in the rat, copulation is readily detected up to 12 hours after the act by the presence in the vagina of the "vaginal plug," formed from the secretion of the seminal vesicles and prostate of the male.

In the case of the mouse this secretion seals the vagina even more completely than in the case of the rat. The plug extends up each cervical canal, and from these right to the vulva, from which it may even protrude. The end is flat, and usually flush with the vaginal orifice, not pointed as in the case of the rat. The removal of the plug is somewhat different in the mouse from the rat. In the former animal the plug becomes softened round the outside, probably owing to the activities of the vast number of leucocytes which are poured into the vagina after copulation. After 18–24 hours this process results in the falling out of the plug almost entire, though the plug may persist in the vagina for 36 hours, or 2 days. Evacuated plugs, coated with cornified epithelium, may on occasion be found lying about the cages, in which case the plug soon becomes desiccated and takes on the appearance of flint. During the stage when the plug is softening it is possible to get smears from round the sides of the plug, but while the plug is firmly in position it is impossible to obtain a smear without a prohibitive amount of manipulation. The smear taken just before the plug falls out consists mainly of spermatozoa and leucocytes, with a few epithelial cells. In some cases the plug may stay in position up to 2 days, and in one case where the vulva was peculiarly constricted a plug was observed to stay in for 3½ days.

Under certain conditions, however, the history of the vaginal plug is slightly different. Copulation may take place at the period of œstrus experienced within a few hours of parturition, when the conditions in the uterus and vagina are very different from normal. For at least a day after parturition a slight discharge, containing large numbers of leucocytes, passes from the vagina, and the plug formed after copulation at this time is far from being the definite structure which is normally found. In many cases coagulation is inhibited and no definite plug is formed, in which event a state of affairs is found which resembles that found during the break-up of the plug in the rat—*i.e.*, the presence in the vagina of an ill-defined mass of débris, of which spermatozoa and leucocytes are the chief components. When parturition has been severe blood is also found. The coagulation of the secretion of the seminal vesicles is probably inhibited by the admixture of lochia. In less pronounced cases a rudimentary sort of plug is formed, but this again only lasts for some 12 hours.

As with the rat, the presence of a plug in the vagina may be taken as an infallible indication of copulation, and, since the act normally takes place at night, inspection each morning will show whether copulation has taken place or not. It is possible, of course, that the technique will miss a small percentage of copulations, owing to premature removal or non-formation of the plug, but in such case the occurrence of œstrus will be indicated by the nature of the vaginal smear.

Histological technique.—After *post-mortem* examination the ovaries and uterus were always preserved, and sometimes the clitoris, vagina, and mammary glands in addition. In the case of uteri which were found in the œstrous state of inflation, removal of a section of the organ results in deflation and consequent loss of the characteristic œstrous appearance. To prevent this two ligatures about 1 cm. apart were put round one horn, and cuts made on the far side of each of these ligatures, a procedure which results in the section being removed in the dilated condition. It is, of course, necessary to exercise care that the tightening of the two ligatures does not increase the dilation of the enclosed section. On account of their small size the ovaries were usually removed complete with the ovarian capsule. The material was fixed in Bouin's fluid and Delafield's hæmatoxylin was used for staining.

Sections were cut at 6–8μ. In cases where the vagina was taken from an animal soon after copulation with the plug still in, very serious difficulty was experienced in cutting, owing to the fact that when dehydrated the plug becomes extremely hard and practically uncutable. No satisfactory means has yet been found to overcome this difficulty.

Identification of animals.—The system of identification in use in the colony is to give each experiment a letter, or letters, and to give each animal a number in addition. The letters are chosen to provide a quick reminder as to the nature of the experiment. Thus the experiments dealt with in this paper have the initial identification letters :—

NVS—normal œstrous cycle.
PVS—vaginal smear during pregnancy.
OVS— „ cycle after ovariotomy.
SOVS— „ „ „ semi-ovariotomy.
LVS—œstrous cycle during lactation.
VP—animals used for following history of the animals after detection of vaginal plug.

Foster-mothering.—Some of the experiments recorded in this paper involved the practice of foster-mothering young from one female on to another. This was necessary where it was desired to find the effect of prolonged suckling. One female will rear 2–3 litters, depending on her condition, during one period of lactation. In actual practice, however, some care has to be exercised to avoid cannibalism on the part of the foster-mother.

Quite apart from experimental work, it is very useful in general breeding work to be able to foster-mother excess young of one animal, or to transfer a litter entire from one female to another. Experience has shown that it can be regularly done with success, if the following precautions are taken :—

(*a*) The foster-mother must not be frightened.
(*b*) The foster litter must be younger than the female's own young. Eating invariably takes place if the foster young are appreciably more advanced.
(*c*) The size of litter must be reduced with each successive lot of young in order to cope with the decreasing food supply.

III. *The Cycle in the Unmated Female.*

Since Allen (1) has dealt in full with the cycle in the unmated female, and since the work recorded here agrees with his account in all essentials, there is no necessity to give a detailed account of the cycles observed in the unmated females. As a control upon the work described later in this paper, it is necessary, however, to consider the normal length of the cycle among these particular mice. Data relating to fourteen unmated females are summarised below :—

Table I.—Length of cycle in unmated female.

Animal.	No. of cycles observed.	Days occupied.	Average length.
NVS 1	4	24	6·0
NVS 2	5	26	5·2
NVS 3	5	28	5·6
NVS 4	3	20	6·7
NVS 6	2	17	8·5
NVS 7	3	19	6·3
NVS 8	2	14	7·0
UVS 1	4	22	5·5
UVS 2	5	26	5·2
UVS 3	7	30	4·3
UVS 4	1	6	6·0
UVS 5	4	28	7·0
UVS 6	7	32	4·6
MUS 1	4	25	6·2
Total	56	317	5·7

This average figure of 5·7 days for the 56 cycles observed compares not unfavourably with Allen's average of 4·5 days. In addition there is reason to suppose, as in the case of Long and Evans' rats, that the first cycles of puberty are protracted at the œstrous stage. The average length of the first cycle observed in three young mice was 7·1 days.

The amount of vaginal content present seems to show cyclic variation. During early œstrus and pro-œstrus it may be impossible to obtain a smear from the vagina. Towards the end of œstrus, however, considerable amounts of cornified material are present, and it is possible to obtain a plentiful smear. In the absence of copulation a gradual infiltration of leucocytes follows this and the amount of smear obtainable remains at about the same level during the meta-œstrus.

IV. *The Cycle in the Mated Female.*

The need for studying the length of the œstrous cycle in does mated with vasectomised males was emphasised by two facts. In the first place, since copulation must be considered as marking the climax of the œstrous period, periodic copulation is the irrefutable test of the occurrence of a cycle, and as such, together with preliminary cornification of the vagina, was the phenomenon to look for in experimental animals. In the second place Long and Evans (5) have shown that in the rat sterile copulation has the effect of materially lengthening the cycle. For this reason the cycle in experimental animals mated with sterile males could not reasonably be compared with the cycle found in unmated females, until the difference (if any) between mated and unmated females had been determined.

It is here proposed to discuss the series of cycles shown by three healthy mature females, which were kept under observation for a very considerable period, 81, 82 and 112 consecutive daily examinations having been made. These animals were respectively known as NVS 12–13–14.

NVS 12.—On the fourth day of observation epithelium only, both cornified and nucleated, was found in the smear. On the next day copulation was found to have taken place. This represented the end of an incompletely observed cycle, and the following seven cycles, all completely observed, may be described in sequence :—

1st cycle. 10 days in length. Nucleated epithelium only was found from the 7th day until the plug was found on the 10th.

2nd cycle. 11 days in length. Leucocytes were present in the smear during the whole time.

3rd cycle. 12 days in length. Some cornified elements appeared in the smear on the 11th day (the day before the plug was found) but leucocytes were still present.

4th cycle. 10 days in length. A few cornified cells were present at the beginning of the cycle from the cornification of the last œstrous period. On the 9th day nucleated epithelium only was found.

5th cycle. 12 days in length. On the 11th day the proportion of nucleated epithelium was high, but leucocytes were still present.

6th cycle. 12 days in length. No pro-œstrous symptoms noticed previous to copulation, except dryness of the vaginal epithelium on the previous day.

7th cycle. 10 days in length. Leucocytes were present right up to the last examination before the plug was found. This animal was then transferred to experimental work.

This animal, therefore, copulated 8 times and had seven complete cycles during the 82 days it was observed. The seven complete cycles totalled in all 77 days, an average of 11 days for each cycle. The small amount of variation (10–12 days) in the cycles is remarkable.

NVS 13.—The first vaginal plug, representing the end of an incompletely observed cycle, was found on the third day of observation. The 8 complete cycles observed were as follows :—

1st cycle : 12 days in length. Nucleated epithelium only was found from the 8th day onwards, and no cornified cells were observed until after copulation.

2nd cycle : 7 days in length. Dryness of the vaginal epithelium was noted at the 6th day, but otherwise no pro-œstrous symptoms.

3rd cycle : 11 days in length. Nucleated epithelium only was found on the 10th day. Cornification was noticed after copulation.

4th cycle : 9 days in length. Leucocytes were present the whole time and no cornification was observed. The vaginal plug stayed in position 2 days.

5th cycle : 11 days in length. During the first day the vaginal plug of the previous œstrus was still in position. On the 10th day nucleated epithelium only was observed. A few cornified cells were found on the day after copulation.

6th cycle: 10 days in length. Leucocytes were present the whole time. No cornification or exclusive presence of nucleated epithelium was observed, but a few cornified cells were found on the day following copulation.

7th cycle: 10 days in length. Dryness of the vaginal epithelium was the only pro-œstrous sign noticed before copulation.

8th cycle: 11 days in length. In this case dryness only preceded copulation.

This female, therefore, spread 8 complete cycles over 81 days, an average of 10·1 days for each cycle. This is slightly shorter than in the case of NVS 12, but the difference is of no appreciable magnitude. The variability in the length of cycle (7–12 days) is, however, noticeably greater than in the case of NVS 12.

NVS 14.—The history of the first 9 cycles of NVS 14 is summarised in the following general observations:—

(*a*) The lengths of the cycles were consecutively 11, 11, 11, 14, 12, 12, 12, 11 and 12 days. It is worthy of note that the longest cycle, 14 days, occurred after the unusual persistence in the vagina for 3 days of the plug from the previous œstrus. Apart from this cycle, the consistency with which 11 and 12 day cycles occurred was remarkable. The actual average is 11·8 days per cycle.

(*b*) On the whole very little cornification was observed, the normal pro-œstrous symptom being the exclusive presence of nucleated epithelium in the vaginal contents.

Conclusions.—From these results on sterile copulation two points need to be considered:—(1) Does copulation take place at the time it would be expected to, from analogy with the rat, and from Allen's work on the uterine, ovarian and vaginal cycles in unmated animals? And (2) What is the reason of the increase in the length of cycle after sterile copulation?

The answer to the first question is undoubtedly in the affirmative. Allen states that during pro-œstrus and œstrus the uterus is greatly distended with fluid, and that mature follicles are to be found in the ovaries. During the meta-œstrus the uterus deflates and no mature follicles are to be found in the ovaries. It will be seen from the foregoing data that copulation is usually detected the day following the disappearance of leucocytes from the vaginal contents and the appearance of nucleated epithelium exclusively.

This time corresponds to the early or mid-œstrous period as assessed by Allen's smear technique, and if it be taken that the ultimate test for the œstrous period is the willingness to copulate, the results as to the time of copulation agree perfectly with Allen's, on the histological detection of œstrus. The fact that in the mated animal the cornified elements are far less in evidence is accounted for by the facts that:—(*a*) On the day when the cornified smear would otherwise be found the plug is in position, and is probably removed with it as a sort of envelope, while (*b*) the remaining cornified elements are removed faster than would otherwise be the case, by the vast number of leucocytes which are poured into the vagina after the formation of the plug.

In certain cases normal animals were dissected immediately copulation was detected, and the organs examined. In these cases the uterus was invariably found to be greatly inflated with fluid, in a manner precisely similar to that described by Long and Evans for the rat. The uterus entirely loses its normal shape, and the lumen becomes positively enormous. In the lumen are found uterine epithelium cells, leucocytes, and a very large number of spermatozoan heads. The ovaries of the animals varied according to the time after copulation at which dissection was made. When the ovaries were obtained soon after copulation had taken place, mature follicles were still visible, but where greater time had elapsed ovulation had taken place and only the ruptured follicles were to be seen. Both these conditions are figured by Allen, and no further illustration need be given here. It is clear, therefore, that the time of copulation adds material support to Allen's valuable vaginal smear work.

Attention may now be turned to the second question.

In the mice mated with vasectomised bucks, 24 cycles were observed in all. These varied in length between 7 days and 14 days, but 21 of the cycles occupy 10–12 days. The 24 cycles occupied in all 264 days, the average per cycle being exactly 11 days. This is almost twice the average length of the cycles in the unmated females, so that it would appear that an effect occurs in mice precisely similar to that which Long and Evans found to occur in rats. The actual results obtained by these authors were: unmated females 5·8 days, mated females 13·1 days. Since mechanical stimulation of the cervix brought about the same result, the authors reasonably conclude that the presence of the vaginal plug stimulated the cervix and led to a condition of pseudopregnancy, and further that this is a means whereby, after fertile mating, the next œstrus is delayed sufficiently to allow the embryo to become well embedded. It is highly probable that the same explanation of the delay of the next œstrus by the presence of the vaginal plug holds good for the mouse.

V. *The Vaginal Smear during Pregnancy.*

When œstrus results in copulation and the formation of a vaginal plug, an extremely large smear is usually obtainable the next day. This is not brought about by dêbris of the plug being still in position, because in the mouse the plug falls out almost entire. The bulk of the smear obtainable after the plug has come away consists of leucocytes, and they are present in far greater numbers than in the meta-œstrus of the non-mated animal. No doubt the irritation caused in the vagina by the presence of a hard mass sufficiently accounts for this increased infiltration. In some cases even the removal of the plug is accompanied by a small discharge of blood.

Percentage copulations resulting in pregnancy.—In considering the history of a female following copulation with a normal male, it is first necessary to know in what percentage of cases, if not in all, the detection of the vaginal plug is followed by pregnancy.

Out of 88 normal females 71 became pregnant after the detection of the vaginal plug. This gives a percentage fecundity of 80·8, and of those failing to proceed to a normal pregnancy some few are known to have been pregnant in the early stages. In such cases, the death and reabsorption of the embryo was only detected by the dissection of the doe when the pregnancy failed to become obvious at the proper time.

Length of gestation period.—It is well known that in rats both copulation and parturition generally take place at night, and since both vaginal plugs and new litters are nearly always first observable in the morning this also appeared to apply to mice. By means of all-night observation it was possible to confirm this view, and as a generalisation it may be said that copulation usually takes place in the early hours of the night and parturition between midnight and early morning. This means that a vaginal plug is probably older when found during a morning examination than a litter is, the difference probably being a few hours. Thus the period of gestation may be taken as the time elapsing between the discovery of the vaginal plug and the discovery of the litter, *plus* a few hours. Of 47 complete gestations recorded, 38 had a period of 19 days, 3 a period of 20 days, and 6 a period of 18 days, so that for this particular stock of mice under the particular conditions existing the period of gestation must be taken as 19 days. This is significantly shorter than the 20 days given by Daniel's (2) observations, and the difference must presumably be put down to differences of race and environment.*

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* Rats kept in the same laboratory have a 24-day period of gestation, 2 days *longer* than that found by Long and Evans.

Vaginal smear during pregnancy.—During the first few days of pregnancy no changes definitely characteristic of pregnancy are apparent in the vaginal contents. During the second week, however, the contents of the vagina acquire a very characteristic nature, and probably differ to some extent from the normal contents in being partly derived from the uterus. A case is dealt with in detail below:—

PVS 1.—This mouse was allowed to have one complete cycle before mating in order that the cornification characteristic of œstrus could be observed in the animal. This first complete cycle lasted 5 days and the animal was subsequently mated to a normal male. Four days after the end of the previous cycle the leucocytes again disappeared from the vagina and, after one day of mixed epithelium and two days of cornified only, copulation took place. Cornified cells disappeared from the vagina two days later and were not found again during the pregnancy. At the beginning of the second week the contents became very viscous, and about the middle of the second week the mucus became tinged with blood. Towards the end of pregnancy, however, the viscosity of the vaginal contents tended to disappear.

Conclusions.—Other animals examined during pregnancy gave precisely similar results, and as a result the following conclusions relating to the vaginal contents during pregnancy may be arrived at:—

(*a*) That, after the first 2–3 days after copulation, when the cornification of the œstrous period is being removed, no cornified epithelium is to be found in the vagina, and in so far as cornification is a criterion of œstrus, it may be said that œstrus does not occur during pregnancy.

(*b*) That during pregnancy the vaginal smear fluctuates between dryness and viscosity, the contents being most viscous about the end of the second week, at which time also a trace of blood may be found in the vagina (coming, in the rat, from the placentæ, according to Long and Evans). Before this time the smear may be stringy, and afterwards, towards the end of pregnancy, the viscosity is less marked. At times the contents are so viscous that it is impossible to get a smear by ordinary means, and if a portion of the viscous contents be pulled out, some difficulty is found in distinguishing different cells among the mucous mass.

It will be seen that this description of the vaginal smear during pregnancy corresponds very closely with that given by Long and Evans for the rat, the chief difference being that the appearance of blood in the vagina is not so localised to certain days as in the case of the rat. Long and Evans' statement, that this "placental sign" of blood is a sure indication of pregnancy, appears, however, to apply with equal force to the mouse.

VI. *The Influence of Lactation on the Œstrous Cycle.*

In several animals lactation has a recognisable effect on the recurrence of the œstrous cycle after parturition. In the rat, in particular, Long and Evans found this to be so. Both the rat and the mouse normally experience a period of œstrus within 24 hours of parturition, but apart from this Long and Evans found the cycle (detected both by vital staining of the ovary and by vaginal smearing) to be completely inhibited so long as suckling was being performed. These conclusions may be briefly summarised for comparison with the data for the mouse to be discussed later. The authors found that:—

(1) Except for the immediate *post-partum* one, ovulation is entirely inhibited during the normal suckling period, and this is associated with a di-œstrous type of vaginal smear.

(2) That œstrus first appears about 7 days after the termination of suckling, 3 weeks after parturition, though the exact time varies according to the number of young which have been suckled. The bigger the litter suckled the longer the time before œstrus reappears. This agrees with Hammond's work on the rabbit (3).

(3) From this they conclude that the non-appearance of œstrus is due to exhaustion of the mother caused by suckling.

(4) When the young are left with the mother œstrus reappears 25–40 days after parturition. Since, however, no mention is made of how long one litter will continue to suckle, this figure is not of much value.

The experiments performed on mice may now be described. All of the females used in these experiments were mated with vasectomised males towards the end of pregnancy, and copulation took place at each œstrous period unless the contrary is stated.

The post-partum cycle in the non-suckling female.—As an essential control on the influence of lactation on the œstrous cycle, it was first necessary to study the recommencement of the cycle after parturition when no suckling was performed, and since, in a number of cases, mating with a strange male during pregnancy caused eating of the young at birth, material for this control was readily obtained. As in the case of the rat, the mouse, of course, ovulates and comes into œstrus within a few hours of parturition, although, as noted above in the section dealing with methods, the detection of copulation is not so easy at this time as is normally the case. The length of the first *post-partum* cycle is therefore reckoned from the time of the detection of this immediate *post-partum* œstrus to the detection of the next œstrus period. Since it was possible that owing to exhaustion or other cause the first *post-partum* cycle might be of greater length than normal even in the absence of suckling, the routine method was to examine these non-suckling control mice till two *post-partum* cycles had been experienced, and in some cases three cycles were followed. The records of these animals are as follow :—

LVS 1.—This female gave birth to a litter of 6, but on the following day the young were found to have been eaten. A vaginal plug was found also on the second day. Fifteen days later copulation occurred again, though the vaginal smears had shown no signs of approaching œstrus. Eleven days after this, on the 28th day of observation, copulation was again found to have taken place, following a vaginal smear of nucleated epithelium only. The next cycle lasted 13 days, the mouse being taken off the smearing after 41 days of observation.

LVS 2 had a litter of 7 early in the evening and vaginal examinations were begun the following day. The second day examination revealed a vaginal plug, and the second copulation took place on the 14th day, the first cycle thus lasting 12 days. Further vaginal plugs were detected on the 26th and 38th days. In this animal, therefore, the three cycles observed were all of the same length.

LVS 3 was mated with a vasectomised male 15 days after copulation with a normal male, but the litter was born dead. The following morning a vaginal plug was found, and copulation subsequently took place on the 15th, 30th and 43rd days of observation. The cycles were thus 13, 15, 13 days in length respectively.

LVS 6 gave birth to a litter of 6 (which, however, was eaten during the day), and copulation took place on the following night. Vaginal plugs were subsequently detected on the 15th and 30th days, the two observed cycles being thus 13 and 15 days in length respectively.

LVS 7 gave birth to a litter of 7, but failed to copulate during the next 24 hours, although mated and showing an œstrous vaginal smear. Presumably, owing to this failure to copulate at the immediate *post-partum* œstrus, the first observed cycle lasted only 5 days, the first copulation taking place on the 7th day of observation. Since the next œstrous period (and copulation) did not occur till the 20th day, this difference was apparently a further example of the difference in the length of cycle in the copulating and the non-copulating female. The third observed cycle lasted 13 days also.

LVS 8.—The litter from this female were dead and half-eaten when found, and copulation had also taken place. Vaginal plugs were also found on the 12th and 24th days of observation.

LVS 10 copulated within 24 hours of the litter of 7 being found. Of this litter 5 were eaten during the first day and the remaining two on the following day. The two observed cycles lasted 12 and 17 days respectively.

LV 17 ate the litter almost immediately, and also copulated a very few hours after parturition. The two cycles lasted 15 and 13 days respectively.

The data for the length of *post-partum* cycles in non-suckling females may be summed up in tabular form, the first cycle of LVS 7 being omitted, as it did not follow copulation and is therefore not comparable with the rest.

Table III.—Length in Days of *post-partum* Cycles in Non-suckling Females.

	No. of cycle.			Average.
	1st.	2nd.	3rd.	
LVS 1	15	11	13	13
LVS 2	12	12	12	12
LVS 3	13	15	13	13·7
LVS 6	13	15	—	14
LVS 7	—	13	13	13
LVS 8	11	12	—	11·5
LVS 10	12	17	—	14·5
LVS 17	15	13	—	14
Average	13·0	13·5	12·7	13·1

Since the average length of cycle in the normal mated animal as described in Section IV worked out at 11 days, it seems possible to conclude from these results that in the non-suckling animal the length of the first three *post-partum* cycles does not vary from the normal for mated animals, and from this basis the problem of the influence of lactation on the length of the cycle may be attacked.

The post-partum cycle in the normally suckling female.—From the above controls it would seem that parturition itself does not influence the length of the succeeding cycles, and thus any disturbance of the cycle during lactation will presumably be due actually to the concurrent lactation, and not to the preceding parturition. In the present sub-section the œstrous cycle in normally suckling mice—that is to say, in mice suckling one litter for the normal period of three weeks—will be discussed.

LVS 14.—This female gave birth to a litter of 7 on the 20th day of observation. The condition of the vagina made it uncertain whether or not copulation took place at the immediate *post-partum* œstrus. The litter was successfully reared and was weaned after 22 days' suckling. During this time no sign of œstrus had been observed, but the day after the litter was removed pro-œstrus set in and copulation took place two days later—*i.e.*, on the 47th day of observation. Nine days later œstrus appeared again.

LVS 15 produced a litter of 8 on the 6th day of observation. The litter was suckled for 22 days and was then weaned. Two days before this cornification of the vaginal contents had been apparent, and a vaginal plug was found at the same time as weaning took place. Copulation occurred again 8 days later.

LVS 21 had a litter of 8 on the 4th day of observation. This litter was weaned 21 days later. Three days before weaning cornification of the vagina was observed, and this persisted for 3 days. No copulation was, however, observed to take place. Six days later œstrus, this time accompanied by copulation, was observed.

LVS 23 had a litter of 9, which was weaned after 22 days' suckling. Pro-œstrus did not occur till 5 days later, however, copulation taking place 29 days after parturition. The next copulation took place 10 days after.

The data relating to the cycle in normally suckling females may therefore be summed up as follows :—

Table IV.—Length of Cycles in Normally Suckling Females.

Animal	Length of cycles.	
	1st cycle.	2nd cycle.
	Days.	Days.
LVS 14	27	9
LVS 18	22	8
LVS 21	21	(6)
LVS 23	29	10
Average	24·8	9

From this résumê it is evident that lactation prolongs the first *post-partum* di-œstrous period to more than double the normal length, the average length of the first cycle in the suckling female being 24·8 days as against 13 days for the first *post-partum* cycle in the non-suckling animal. As regards the second cycle, it should be remembered that the second cycle of LVS 21 was not preceded by copulation, and is therefore not comparable with the others. The remaining second cycles, however, are shorter, if anything, than the normal, so that the increased length of the first cycle is not reflected in the second one. In other words, the return to normal is abrupt, not gradual.

The cycle during prolonged suckling.—In certain cases discussed in the last paragraph, it was noticed that pro-œstrus set in before the removal of the litter, and this suggested that lactation is no actual bar to the occurrence of œstrus. It thus became of interest to examine the effect of prolonged suckling produced by applying foster-young after the weaning of one litter, in order to ascertain whether the second cycles in such cases would partake of the nature of the first long lactation cycle or would be of the normal length.

LVS 11 had a litter of 6 on the 20th day of observation. This litter was weaned 21 days later, and 6 new-born young were substituted and were adopted by the mother. One day after this copulation took place. Pro-œstrus, however, did not occur again for 13 days, and not for 15 days was copulation detected. The foster-litter was successfully reared and was weaned some days later.

LVS 16 had a litter of 7 on the 20th day of observation and copulated the following night. This litter was weaned after 21 days, and was replaced by 5 young of 14 days old. The first œstrus was detected 30 days after parturition. The day following this the second litter was weaned and 7 new-born young substituted. Though not suckled well, the third litter survived, and no further copulation occurred till 45 days after parturition.

LVS 9 had a litter of 10, which were weaned after 22 days' suckling, a litter of 6 at 2 days old being substituted. The foster-litter was adopted and reared. The first copulation took place 23 days and the second one 40 days after parturition.

LVS 4 suckled three litters consecutively in all, lactation proceeding continuously for 48 days. During this time two cycles were observed, the first one having the excessive length of 37 days, while the second was normal at 11 days.

LVS 5 had a litter of 11, and subsequently suckled another, lactation being continuous for 40 days. The two cycles observed occupied 25 and 13 days respectively.

These data relating to the œstrous cycle under conditions of continuous suckling may be summarised as follows :—

Table V.—Cycles in Prolonged Suckling Females.

Animal.	Length of cycles.	
	1st cycle.	2nd cycle.
	Days.	Days.
LVS 4	37	11
LVS 5	25	13
LVS 9	23	17
LVS 11	22	15
LVS 16	30	15
Average	27·4	14·2

The average length of both first and second cycles is thus longer than in the case of the normally suckling group, but not to any significant extent. The first cycle average (27·4 days) is very near that for the first *post-partum* cycle in the previous group, and, in fact, serves to confirm the conclusion that lactation considerably lengthens the first cycle. The above table, however, makes it quite clear that this effect is not extended to the second cycle, even when lactation is experimentally prolonged. The average length of the second cycle where suckling is prolonged (14·2 days) is only slightly longer than that for the normal mated female of 11 days, and practically identical with the average length of the second *post-partum* cycle in the non-suckling female.

Conclusions.—The following conclusions may be drawn from this work on the effect of parturition and lactation on the œstrous cycle :—

(1) Normal œstrous cycles succeed parturition immediately in the absence of suckling.

(2) When the doe is allowed to rear her litter the first di-œstrous period is greatly lengthened. The œstrus following on parturition occurs immediately, but more than 3 weeks elapse before the next œstrus appears—*i.e.*, it appears at about the same time as or just after the litter is normally weaned. The second cycle is of normal length, even when prolonged suckling is induced by applying foster-litters.

The chief point requiring explanation is thus the length of the first *post-partum* cycle in the lactating doe. Is there actually some inhibitory mechanism, or is it merely a quantitative relation ? It is possible that lactation may have some reflex action on the *corpora lutea* of lactation, causing them to persist and inhibit œstrus longer than the *corpora lutea* of ovulation. In actual fact they do persist longer and become larger, but is this due directly to a stimulus from the mammary glands ? The fact that, if the young are removed, œstrus appears at the normal time, suggests such an explanation.

As regards the rat, Long and Evans (5) detected changes in the lutein content of the *corpora lutea lactationis* within 24 hours of the cessation of lactation.

In an endeavour to throw light on this problem, certain females were left with only one or two young after parturition. This resulted in a first cycle of the type found in the non-suckling doe, and therefore, as far as œstrus is concerned, the suckling of only two young is not sufficient to produce the inhibiting stimulus. One is reduced, therefore, to supposing that the stimulus of lactation causes growth and persistence of *corpora lutea* formed after the immediate *post-partum* ovulation, and that these in turn inhibit the next œstrus for 3 weeks or more. Further, one must suppose that this lactation stimulus is of a purely quantitative nature, the suckling of an appreciable number of young being necessary for its production, and that prolongation of lactation beyond a certain point has no further inhibitory action.

VII.—*The Œstrous Cycle after Unilateral Ovariotomy.*

Complete removal of both ovaries obliterates the cyclic changes from the whole female genital tract, and the vaginal smear after double ovariotomy (in the absence of ovarian regeneration) becomes a mixture of leucocytes and nucleated epithelium (mostly leucocytes, in which no cornified cells can be observed), and differing from the normal di-œstrous smear by being serous rather than milky.

Unilateral ovariotomy has, of course, no such effect, and the following records show the length of cycles after unilateral ovariotomy. In each case the operation had been performed during pregnancy for a different experiment, and the vaginal smearing was begun some two months afterwards, each doe being mated separately with a vasectomised buck. Since the œstrous periods were in most cases quite normal, it is unnecessary to describe the animals individually, and the following table sums up the results :—

Table VI.—Œstrous Cycle in Unilaterally Ovariotomised Does.

Animals.	No. of œstrous periods.	No. of copulations.	No. of complete cycles.	Length of cycles.	Average length.
SOVS 1	3	3	2	12–11	11·5
SOVS 2	3	3	2	15–15	15
SOVS 3	3	3	2	10–12	11
SOVS 4	3	2	2	23–13	18
SOVS 5	4	3	3	13–16–13	14
SOVS 6	3	0	2	8–9	8·5
Total	19	14	13	170	13·0

SOVS 6 was in bad condition (when killed it was found to have gut adhesions), and this fact probably accounts for the failure to copulate. With the exception of SOVS 6, each animal in this series had the remaining ovary removed, after the cycles summed up in the above table had been detected, and the immediate cessation of the cycle showed that the production of regular œstrus had not been assisted by the presence of regenerated ovarian tissue on the site of the first ovariotomy. It would seem, therefore, that removal of one ovary has little effect on the œstrous cycle.

VIII.—*Summary.*

(1) The average duration of the œstrous cycle (meta-œstrus to œstrus inclusive) of the unmated albino mouse in the colony maintained at University College, London, was found to be 5·7 days.

(2) The average duration of the cycle in normal females mated with vasectomised bucks was found to be 11 days. This postponement of the next œstrous period is considered to be due to the formation of a decidua by the uterus, on mechanical stimulus by the vaginal plug, a process which has been demonstrated in the albino rat by Long and Evans.

(3) Pregnancy follows the detection of the vaginal plug in about 80 per cent. of cases, the length of the gestation period being 19 days in almost all cases. During pregnancy the vaginal smear shows no cornified material. About the 10th–12th day blood appears in the vaginal mucus, called by Long and Evans the "placental sign" in the case of the rat.

(4) The effect of lactation is entirely to inhibit œstrus (apart from the immediate *post-partum* period) for at least 3 weeks. This result is, however, only brought about where an appreciable number of young are suckled. Rearing of one or two young does not prevent the return of œstrus. After the inhibition due to suckling the cycle goes suddenly back to normal, even in cases where lactation is prolonged.

(5) Unilateral ovariotomy does not appear to influence the cycle.

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Additional Note on the Effect of Insulin on the Lactacidogen Content of the Skeletal Muscles.

By C. H. Best and H. P. Marks.

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It has recently been shown (Best, Hoet and Marks, these *Proceedings, supra*, p. 55) that in the spinal preparation to which sugar is administered intravenously, no significant portion of the dextrose which disappears under the action of insulin is stored as a phosphate ester in the muscles. In these experiments the inorganic phosphorus of the blood was greatly reduced but there was no depletion of the free phosphorus of the muscles. The possibility remained that although an abundance of phosphate appeared to be available in the muscles the amount of inorganic phosphorus present in the blood might exert some effect on the formation of lactacidogen. We have therefore carried out experiments in which the inorganic phosphorus of the blood has been maintained at a high level by the intravenous administration of sodium phosphate. Dextrose and phosphate, in approximately the proportion that they are present in hexose diphosphate, were infused in mixed solution. The results of one of the experiments are given below.

Cat, 4,100 grams. Spinal preparation. Not eviscerated.

	Before insulin.	*At end of experiment.*
Blood Sugar	0·38	0·23 per cent.
Glycogen, av. of 4 muscles ..	0·768	1·032 ,,
Inorganic phosphorus of blood	6·9	21·3 mg. per 100 c.c.
Free phosphorus of muscles ..	95	99 mg. per 100 grams.
Combined phosphorus of muscles	144	142 ,, ,,
Lactacidogen	49	43 ,, ,,

The results of these experiments show that even when the inorganic phosphorus of the blood is kept at a high level there is no storage of dextrose as a phosphate ester in the skeletal muscles. The increase in glycogen is of the same order as that obtained in similar experiments in which phosphate was not administered.

On the Occurrence of the Œstrous Cycle after X-Ray Sterilisation.—Part I. Irradiation of Mice at Three Weeks Old.

By A. S. Parkes, Beit Memorial Research Fellow.

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(From the Dept. of Physiology, University College, London.)

(Plates 3–5.)

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I. *Introduction.*

The cyclic activities of the female reproductive organs of mammals, constituting the œstrous cycle, fall naturally into two main series, *i.e.*, the uterine cycle and the ovarian cycle, and the correlation between these two forms one of the most intricate problems of internal secretion. Two chief problems are presented—(*a*) what stimuli produce the uterine cycle in the first place, and (*b*) how is the uterine cycle so synchronised with the ovarian cycle as to result in the uterus being ready to receive the fertilised ovum just after the time of ovulation ? Since ovariotomy results in cessation of the cycle, whereas excision of the other reproductive organs has not this effect, it is necessary to suppose that the controlling power resides in the ovary, and further, since transplantation of the ovary does not result in the cessation of the cycle, that the means of control is hormonic. The alternating maturation of follicles and *corpora lutea* in the ovaries is exactly synchronised with the changes which take place in the uterus, and it is therefore reasonable to look to the periodic growth of one or other of these ovarian structures for the origin of the periodic hypertrophy of the uterus. Owing to the fact, however, that in many mammals œstrus sets in when no *corpora lutea* are present in the ovary, these structures can be eliminated as originators of the œstrous cycle. Similarly, the supposition that the *corpus luteum* has an inhibitory effect, the removal of which allows œstrus to appear, can only be applied to certain special conditions. If, therefore, any cyclic structure of the ovary is responsible for the regulation of the œstrous cycle, it must be the maturing follicle. This conclusion is supported by the following facts:—

(*a*) Œstrus first appears when ovulation starts at puberty, and the œstrous cycle stops when ovulation ceases at the menopause.

(*b*) In animals with a restricted breeding season the annual beginning and end of the cyclic activity of the uterus coincides with the beginning and end of ovulation.

(*c*) In the ferret and the rabbit, where ovulation is not spontaneous, the mature follicles and the condition of œstrus persist together for some time in the absence of coitus.

(*d*) In the cow, when large follicles become cystic, a nymphomaniac condition of persistent œstrus may result.

(*e*) During pregnancy no follicles normally mature and neither physiological nor psychic symptoms of œstrus occur.

The importance assigned to the part played by the maturing Graafian follicle in the causation of œstrus is well shown in the following citations from the recent writing of authors of established repute.

Speaking of the œstrus-producing hormone Allen (2) says: "Its presence and absence, due to the periodic development of successive sets of follicles, is sufficient to explain the mechanism of œstrous phenomena." In a later paper Allen and collaborators (5) say: "From a functional analysis of the follicle through its various stages of growth it seems probable that the production of this hormone is referable ultimately to the metabolism of the ovum itself as the dynamic centre of the follicle." Hartman (13) says: "What element of the ovary constitutes the source of the stimuli which lead to pro-œstrum and to œstrus? The opossum affords an unequivocal answer, which is in full accord with the clear and succinct statements by both Allen and Robinson, in which they make out a case for the Graafian follicle. Their reasons I consider conclusive." Robinson's (24) work on the ferret led him to conclude that: "The phenomena of pro-œstrum and the œstrus only appear when a group of follicles has attained a stage of development which may be called pre-inseminal maturity, and the phenomena are due to some secretion produced by the follicles in that phase of their development." In a very recent paper, Zondek and Aschleim come to the conclusion that the œstrus-producing hormone is elaborated by the theca cells (28).

It must be admitted, however, that the theory that the maturing Graafian follicle is responsible for the onset of œstrus has resulted largely from the elimination of other probabilities, and not from an experimental basis. In fact, the only experimental work which appears to have been carried out on the problem gave conflicting results. Marshall and Runciman (15) were unable to stop the onset of œstrus in the dog by rupturing all maturing follicles just before the period was due. This result suggested that the presence of mature follicles was not essential for the production of œstrus. Marshall and Wood (16) later report, however, that they were unable to confirm these results. Since, however, the operation of laparotomy and follicle puncturing is somewhat severe, it is not improbable that post-operative effects alone might inhibit œstrus, especially as regards psychic manifestations. Furthermore, if the ruptured follicles produced luteal tissue, this might very well result in the suppression of the œstrous period.

Considerable support of the view that the Graafian follicle acts as an organ of internal secretion and produces a hormone causing heat is to be found in the experiments relating to the production of heat in ovariotomised mice by the injection of liquor folliculi extracts. Many papers have been published during recent years on these lines, but the main credit belongs to Allen and Doisy (3–4–5–6), who, in a series of papers published during the last three years, have given detailed accounts of the preparation and action of extracts of liquor folliculi. By repeated extractions with alcohol and ether, a fatty residue can be obtained which, when injected into ovariotomised animals, results in the appearance of œstrous symptoms.

Not only are these experiments of Allen and Doisy's well controlled and critically examined, but they stand out in marked contrast to the majority of experiments of this nature, which are mostly only indifferently controlled or even entirely uncontrolled. There appears to be one possible criticism only, and that relates to the test animals. Ovariotomised mice (used by Allen and Doisy as test animals) are reported by Davenport (10) to regenerate ovaries in some 60 per cent. of cases within 3–16 weeks after operation. That this striking state of affairs does exist has been amply confirmed by the present writer, working in collaboration with Dr. Brambell, and it is certain that ovariotomised mice may very quickly re-form ovarian tissue, even after the most careful surgical removal of the original ovaries, capsule, and fat body. This means that no ovariotomised mouse should be used for testing extracts for more than 2–3 weeks after the operation, unless steps are taken to show that no regeneration has taken place.

At the same time it is in the highest degree improbable that any large proportion of their positive results could have been brought about by regeneration of ovaries, and their experiments, which have been amply confirmed by Dodds, Dickens, and Wright (11), and also by the present writer working in conjunction with Mr. C. W. Bellerby (21), show conclusively that a substance, either fatty in nature or at least associated with fats, which is capable of producing the histological and physiological symptoms of œstrus in ovariotomised females, can be extracted from liquor folliculi. However, while it is certain that such a substance can be extracted from follicles, is it certain that it is produced by the follicle during its later stages of growth ? In other words, is a maturing follicle an essential forerunner of œstrus ? The experiments recorded in this paper were performed in an endeavour to answer this question.

As is well known, the Graafian follicles are entirely destroyed by prolonged exposure to X-rays. Halberstadter (12), in 1905, first reported atrophic changes in the ovaries of rabbits as the result of exposure to X-rays. The irradiated ovaries were smaller than the normal, and showed complete disappearance of Graafian follicles in the case of those receiving long exposures. The same results on the same animal were found by Bergonié, Tribondeau and Recamier (7). The *corpora lutea* were found not to be affected. Bouin, Ancel and Villemin (8) showed that the interstitial tissue remained intact, and Houssy and Wallart (14) found increase of the interstitial tissue in a human ovary irradiated incidentally to treatment for uterine myoma. These authors have been confirmed in their main conclusions by many other workers, reference to whom is made by Colwell and Russ (9).

It is clear that while all authors agree that the follicles are destroyed by prolonged exposure to X-rays, there is considerable doubt about the effects on the development of the accessory organs of reproduction, and it was while work (of which an account is now being published) was in progress on this problem that the observation was made which led to the experiments described here. Some X-rayed females were being dissected for microscopical examination, when it was noticed that the uterus of one was in a typical œstrous condition, and this appearance was confirmed by an examination of the vaginal contents. Since sections of the ovary showed the complete absence of follicles, vaginal smearing was started on the rest of the same batch of females, and further animals were irradiated for the purpose of ascertaining whether destruction of the Graafian follicles by X-rays in the young animal did, or did not, inhibit the œstrous cycle when the animal subsequently became adult.

II. *Methods and Material.*

The technique of irradiation, the management of the animals so far as it concerns these experiments, together with the methods used for detecting the œstrous cycle, are discussed below.

Management of animals.—The management of the colony has been fully dealt with elsewhere (20), but the following details are relevant here. The mated females are examined daily, and when a vaginal plug (indicating copulation, see later) is found the animal is segregated. If, as is usually the case, pregnancy ensues the sex of the young in the litter is noted, and the number suckling is reduced (if necessary) to 4–6 according to the age and condition of the doe. When the young are three weeks old the mother is removed, but the young are left in the nest for a further week or fortnight until they get over the disturbance due to being weaned. The two sexes are then separated and the young are removed to large rearing cages. This system has been found to produce the best and least interrupted growth of young. At 10–12 weeks old, according to condition, those animals destined for normal breeding are mated. The experimental animals described in this paper were treated in exactly the same way as the normals, except for irradiation, and except for the fact that in some cases where it was not desired to irradiate the males, the females were separated at weaning.

Dosage of X-rays.—As a result of a large number of experiments (some of which have already been reported) on irradiating animals of both sexes at all ages with varying doses, an intensity given by the following constants has been found to produce the desired results without unreasonably long exposures:—

Kilovolts, 50.
Milliamps, 5.
Wave-length, 0·285 Å.
Spark gap (point-point), 6·3 cms.
Tube—Coolidge universal, medium.
Distance from anti-cathode, 18 ins.
Filter, none.

The original exposures given were of 10 minutes duration, but neither male nor female at any age were found to be sterilised by such treatment, though as reported elsewhere (Parkes 17) such a dosage applied to the male was found to affect the sex-ratio. For sterilisation purposes the exposures were gradually increased to 80 minutes, and such irradiations, given in two exposures of

40 minutes each, were found to be sufficient to sterilise females with perfect regularity. This dosage was found to give approximately $\frac{1}{4}$ B tint with Levi's pastilles. It may be mentioned here that the criteria of sterility as applied to these animals are:—

(1) Incapacity to breed when mated with normals of the other sex.

(2) Histological absence of gametes on microscopical examination of gonads.

The first animals of the series dealt with in the present paper, *i.e.*, the females of three litters,* were given one exposure of 40 minutes when weaned at 3 weeks old, but (as explained later) this gave uneven results as regards sterilisation, and subsequent animals in this series were given a second exposure of the same duration.

After treatment.—After irradiation sample mice were taken at various intervals and autopsies performed to ascertain the effects of the X-rays. The rest when adult were mated with normal males, and each pair kept separately. After sufficient time had elapsed to test their fertility and œstrous cycle, most of the animals were killed, and the ovaries, mammary glands (where possible), uterus and clitoris preserved for histological examination. In view, however, of Davenport's work on the regeneration of ovaries in mice it became of interest to ascertain what sort of gonad, if any, these sterile mice would regenerate after ovariotomy. For this reason, in certain cases the ovaries were secured for histological examination by double ovariotomy, and the animal was otherwise kept intact, the vaginal smearing being continued as before. In certain of the mice subjected to the larger exposures, the growth during the 4th–12th weeks was extremely bad, and some of the worst cases were very reminiscent of the condition of cretinism. Since it thus seemed possible that the thyroid had in such cases become deranged as a result of the irradiation,† thyroid feeding was resorted to in the case of the most abnormal young. Since, however, it seems probable that some correlation exists between the thyroid and the gonads, the administration of thyroid was very sparing, thyroideum siccum being shaken up with the milk in which the bread was soaked in sufficient amounts to ensure that each animal consumed about 1 mg. per day. This treatment did seem to result in an improvement in the young, so that apparently thyroid deficiency did exist. This problem is being further investigated.

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* These three litters were numbered respectively M G X 1, M G X 2, and M G X 3, the capital letters indicating the experiment and the number indicating the litter. The individual females were given a small letter *a*, *b*, and so on. Thus, M G X 1*a*, M G X 1*b*, etc. Later, however, this system was simplified by giving each female the capital letters denoting the experiment and a number only, thus M G X 21, M G X 22, and so on. These identification numbers will be referred to in subsequent publications, but in the present paper, since all the mice belong to the same series, they will be referred to by letter M and their numbers only.

† Since irradiation is used therapeutically to reduce exophthalmic goitre, such a result would not be entirely surprising. Rave's (23) review of work on the histological results of irradiations of thyroid seems, however, to suggest that only negative results have been obtained.

Histological technique.—The methods employed for dealing with the microscopical examination of the reproductive organs have been fully described in another paper on the normal œstrous cycle. Since precisely the same methods were used in the examination of the irradiated material, further description is unnecessary here.

Detection of the œstrous cycle.—The œstrous cycle examinations were made on the basis of the vaginal smear technique, first applied to the mouse by Allen (1). Since this technique has often been described, and is discussed in another paper (Parkes, 18), no detailed description need be made here.

Allen found that the length of the cycle was normally about 4–6 days in the unmated female, but this varied very considerably, as also did the length of the component stages of the cycle. The range for the length of the complete cycle was 2 days to 16 days.

Since the di-œstrous period usually lasts two days and the other three periods about one day each, it follows that although each period will probably be detected, examination once a day, as in the case of the present material, may not detect the finer graduations. Thus the pro-œstrum and the first part of the œstrous period (when the epithelium may be dry) may not both be detected in the same cycle by 24 hourly examinations.

Since Allen states that considerable variation is found in different stocks of mice, and in view of the importance of this technique from the point of view of the present research, it was deemed advisable to check the normal œstrous cycle in normal animals in the same colony as the experimental mice were drawn from. With the reservation made above relating to the fact that only one examination a day was made, it may be said that my results on the unmated animal entirely bear out Allen's work, and further strengthen it by the introduction of sterile copulation, which definitely marks the physiological œstrus without the complication of pregnancy. In the mouse and the rat copulation can be detected for 12–24 hours afterwards by the presence in the vagina of the "vaginal plug," formed from the secretion of the seminal vesicles. Thus, by mating females with a vasectomised male and making smears from the vagina, an exact check can be kept on the œstrous cycle.

These observations are being published elsewhere, but for comparison with the experimental cycles it may be said that the average length of the normal cycles observed in mated females was 11 days. As a purely arbitrary arrangement one cycle is considered to run from the end of one œstrous period to the end of the next. Thus, in the mated animal, copulation is taken as the end of the cycle, and the smear for the next day begins the new cycle, which in turn will be concluded by copulation. In the case of the unmated or non-copulating female, the termination of the pure cornified smear is considered to be the end of one cycle and the beginning of the next.

III. *Experiment Records.*

The successive batches of animals dealt with in this paper are as follows. In the first place three litters (M 1, M 2, M 3), of which only the females are dealt with in this paper, were irradiated for 40 minutes, at the standard intensity detailed above, when the young were 3 weeks old and had just been weaned. Of the first litter 4 females were reared, of the second 4 females also, while of the third litter only two females lived to maturity. These ten females were being killed for *post-mortem* examination at 10 weeks old, when the condition of the uterus of M 1*b* (Plate 4, fig. IIID) suggested that the œstrous cycle was intact, and as a result those not already killed (five) were kept for vaginal smearing and mating. *Post-mortem* examination of those already killed, M 1*a* and *b*, M 2*a* and *b*, and M 3*a*, revealed very small ovaries, but well-developed accessory organs and microscopically complete absence of follicles and *corpora lutea* in the ovaries. The histology of the gonads of these five females, together with that of the ovaries of the other completely sterilised females, is dealt with in general in the next section.

These results suggested that the ½ B tint exposure employed was sufficient to sterilise females at weaning time, and it was confidently expected that the remaining five would likewise be sterilised. This expectation, however, was not realised. The individual histories of these animals (M 1*c* and *d*, M 2*c* and *d*, and M 3 *b*) are as follows. M 2*c* died after having vaginal smears taken for four days, an event which was not altogether unexpected, since the animal was in very bad condition. M 1*c* showed signs of having a cycle and was mated with a normal male. Copulation took place two days later, and a litter was produced after a gestation period of normal length. This animal had therefore escaped sterilisation and is of no further interest.

Of the remaining three, one (M 1*d*) was completely sterile and will be dealt with later. The other two both had cycles, but on *post-mortem* examination neither was found to be quite sterile. M 2*d* during the 30 days under observation was observed to have four perfectly normal cycles, averaging a little over 7 days each in length. She was mated with a normal male after the first cycle, and coitus took place at the œstrous period of each of the three succeeding cycles. At the end of the fourth cycle the animal was killed. The *post-mortem* examination showed the ovaries to be small, but a typical condition of œstrous dilation existed in the uterus. Histological examination of the ovaries revealed no trace of mature or immature follicles and no ova in the left ovary, but the right ovary, in spite of the fact that the main body showed a condition similar to the left ovary, contained a structure which could only be considered as a *corpus luteum.* The last œstrous period was thus presumably accompanied by the maturation of a follicle (only one) and the discharge of at least one ovum. No other *corpora lutea,* however, were to be seen in the ovary, and since the previous œstrous period had occurred only 6 days previously, old corpora from this period, as well as from the one before, should have been present if ovulation had taken place at these previous periods. The continued sterility of the animal also suggests that no other ovulation had taken place. In spite, however, of the evidence that this mouse had had at least two periods of œstrus without coincident maturation of follicles, the *corpus luteum* found makes it necessary to dismiss this case, in order to confine the series to be discussed in the next two sections of this paper to completely sterilised animals.

M 3*b* was observed for 32 days, during which time two complete cycles, averaging 14 days each, were observed. Both periods of œstrus resulted in coitus. On microscopical examination of the ovary it was discovered that the right ovary was quite free from both follicles and *corpora lutea,* but the left ovary contained one small *corpus luteum.* Here, again, the absence of other *corpora lutea* seems to preclude the possibility of ovulation having taken place at the first observed periods of œstrus, but for the reasons explained in the case of M 2*d*, the presence of the small *corpus luteum* makes it necessary to dismiss the animal also from the series.

It is curious that while the first five animals examined should be completely sterilised, out of the second five one was quite fertile and two not completely sterilised. There can, however, be no possibility that the re-formation of oocytes accounts for this. The period between the two sets of *post-mortem* examinations was in no case more than 5 weeks, and it is scarcely likely that if no re-formation had taken place in the first 7 weeks after irradiation, complete re-formation would take place in the next 5 weeks. As a matter of fact, M 1*c* conceived within a fortnight of the first five being shown to possess no ova or follicles, and in the case of M 2*d* and 3*b* no signs of re-formation were found, only the luteal tissue already described.

The probable explanation of the unequal distribution of the non-sterilised animals between the first and second groups is that the smallest and poorest mice were picked out for autopsy first, and that such animals were those most affected by the X-rays. However, whatever the reason for this, the fact remains that the dosage employed had not sufficed to sterilise all the young animals on, and it was decided to treat a further batch of young with double the dosage. Accordingly, ten female young at 3 weeks old were given 40 minutes' exposure, and the irradiation was repeated a week later. These young were from two litters, six (M 6*a–f*) from the first and four (M 7*a–d*) from the second. Of these M 6*d* and M 7*b* and *d* died before reaching maturity, and in these cases *post-mortem* putrefaction had proceeded too far to allow of accurate examination. The remaining seven animals, however, arrived at maturity and were dissected after varying periods of vaginal smearing and mating. M 6*a* was peculiar in getting prodigiously fat, as shown in Plate 5, fig. V. When dissected the body cavity was found to be absolutely full of fat, which totally obscured ovaries, uterus, kidneys, mesenteries and much of the small intestine.

The remaining animals, however, were externally fairly normal. Six of these seven animals copulated at least once, and some of them several times, and since no young were ever produced the ½ B tint exposure appeared to have been effective in sterilising the ovaries. Microscopical examination confirmed this supposition. In none of the ovaries were any ova to be found, and the absence of any true *corpora lutea* seemed to dispose of the possibility of ovulation having taken place. These animals will be considered in detail in a further section of this paper.

Since the results on these animals seemed worthy of repetition, a further 20 young females (numbered M 8–27) were irradiated at 3 weeks old. The subsequent growth of many of these young was bad, but eventually they all became fair-sized adults, with the exception of M 15 and 16. M 19 was dissected before the vaginal smear examinations were begun, and proved to be completely sterile. Œstrous cycle histories are thus available for M 8–14, 17–18, 20–27, making with those of the previous two batches 25 cases of completely sterilised animals in all. The œstrous cycle histories of these animals will all be dealt with in Section VI.

M 28–35 were males irradiated at weaning time for the purpose of ascertaining the influence of the absence of germ cells on the development of the accessory organs of reproduction. M 36–42 were females irradiated at the same time and (with the exception of 42) dissected at intervals of a week for the purpose of tracing the changes taking place in the irradiated ovary. The account of the histology of the irradiated ovary given in Section IV is founded largely on this series.

IV. *Histological Effects of Irradiation of the Ovary.*

The ovary of the adult animal sterilised at weaning time consists of a mass of tissue of a parenchymatous nature, interspersed with cavities which possibly represent the original location of the follicles. These cavities usually contain varying amounts of eosinophilous matter, which appears to be entirely necrotic. One such typical sterilised ovary is shown in Plate 3, fig. IIA, the ovary in question being that of M 1*d*. The derivation of the mass of tissue which makes up the ovary is not obvious from the examination of adult material, and also it is impossible to say from such a section whether or not the necrotic material contained in the cavities represents the whole of the original follicle or merely some part of it. The point is of importance, since the persistence in the ovary of tissue of follicular derivation would complicate any explanation of the occurrence of the œstrous cycle in the sterilised female. It thus became imperative to trace the transformation of the irradiation ovary, and for this purpose animals were dissected weekly, for a period of six weeks after irradiation. This material, together with that secured from previous adults, forms the basis of the following account (prepared in collaboration with Dr. F. W. R. Brambell and Miss Una Fielding) of the changes in the irradiated ovary. The joint paper which it is hoped to publish shortly on this subject will contain a more detailed account of these changes.

The first effect of irradiation is to produce degeneration of all small oocytes, and this is followed by maturation and segmentation changes in the older oocytes. The follicle cells and theca interna elements, on the other hand, do not show this rapid hypertrophy. This growth and segmentation on the part of the oocytes is, however, pre-degenerative.

The ova then degenerate and are absorbed first; but, finally, both follicle and theca interna cells themselves degenerate and eventually disappear. They are, however, never observed to pass out of the theca externa. While these changes are proceeding a new growth of tissue is invading the stroma and penetrating between the degenerating follicles. There is every reason to believe that this new growth does not originate from granulosa or theca interna elements. It has the appearance of coming from the periphery, the probability being that the germinal epithelium is the site of origin. Within a few weeks after irradiation this tissue comes to form the entire ovary and is arranged in cords surrounded by slight fibrous sheaths. The cells of the cords gradually enlarge and in many cases become loaded with fat.

Gradually there sets in a second formation of cords, which may be more or less continuous in origin with the first proliferation, or may, on the other hand, be distinct. This second formation originates from the germinal epithelium alone, and each cord is surrounded by a much more definite fibrous sheath than is the case with the first formation. As they grow from the periphery inwards and as more are formed, the tissue which has resulted from the first proliferation is pushed into the centre of the gonad, in precisely the same way as are the medullary cords of the embryo. In M 6*e*, which had been left for 18 weeks after irradiation, these cords had definitely become tubes, which have the appearance of being of a spermatic nature. In these cords cells are arranged as a single lining layer, surrounding a number of free cells within the lumen. In M 12 the cells of the second proliferation of cords exhibit many mitoses and are somewhat similar to spermatogonia.

Variable numbers of cords in various stages of development have been found in practically all the irradiated ovaries examined, and the appearance of cords of both the first and second invasions is not inhibited by the presence in the ovary of atretic follicles, or by the presence of what at first sight seem to be small spurious *corpora lutea* formed by atretic follicles.

This general account describes the main features of the sterilised ovary, and though many points are obscure, it is clear that with the dosage used for M 6–27 complete destruction of ovary is attained within 6 weeks of irradiation —that is to say, by the time the animal has become mature. The second fact of importance from the standpoint of the present paper is that in the great majority of cases no tissue of follicular derivation is present in the ovary by the time the irradiated animal has become adult. Individual idiosyncrasies in the ovaries will be mentioned in the section dealing with the œstrous cycle in the sterilised animals.

V. *Effect of Sterilisation on the Development of the Accessory Organs of Reproduction.*

The discovery that X-rays could conveniently be used to destroy the gametogenic portions of gonads soon led to their use in experiments designed to throw light on the site of formation of the hormone responsible for the development of the accessory organs of reproduction and of the secondary sexual characters. The point at issue, of course, is whether this hormone is produced by the part of the gonad producing germ cells or by some other tissue (the interstitial tissue of the testis and the inter-follicular tissue of the ovary). Many authors have found that sterilisation of the adult by irradiation does not lead to degeneration of the accessory organs in the same way as gonadectomy is known to, but the critical test is obviously, " Do the accessory organs develop normally after sterilisation of the young animal ? " The small amount of evidence available on this point suggests emphatically that the development of the prostate, seminal vesicles, etc., of the male is not interfered with by destruction of the seminiferous tubules.

From the work recorded in this present paper, and from much as yet unpublished material, considerable data have been collected relating to this question, and it may be said with confidence that complete destruction by X-rays of the gametogenic tissue of the gonads of either sex, before birth, at birth, or at weaning time, has no inhibitory effect on the development of the accessory organs of reproduction of the mouse. Unfortunately, the mouse has no recognisable secondary sexual characters, so that the generalisation cannot as yet be unequivocally extended to these characters, though little doubt can be felt that they would follow the same law. It is proposed to publish the detailed observations on this subject as soon as practicable (19).

VI. *The Œstrous Cycle after Sterilisation.*

The first sign that sterilised females might have cyclic activity in the accessory organs when adult was first provided by M 1*b*. This female was one of those originally killed for histological examination, and was, as mentioned above, quite sterile. At the time of dissection it was noticed that the uterus was distended with fluid in the manner characteristic of the œstrous period, and at the same time the vaginal smear consisted solely of nucleated epithelium, again an indication of œstrus. Since a sterilised female would probably not be in a perpetual state of œstrus, some sort of cyclic activity was suggested.

Altogether the œstrous cycle histories of 23 animals known to be sterilised are available. In addition, 2 animals (M 14 and 24) are assumed to be sterile, since 25 others of the same batch were. As, however, neither of these animals exhibited cyclic activity of the accessory organs, a condition also shown by

6*c*, they have not yet been examined, as it is hoped that a pronounced development of tubules is present, as in the case of M 6*e*.

The œstrous cyclic records of these 25 animals are as follows :—

M 1*d*.—Was observed for 37 days, and during this time the vaginal contents went through the changes characteristic of œstrus four times, giving three complete cycles observed. The respective lengths of these three cycles were as follows—5, 13, 12 days, the average length being 10 days. In addition, two part cycles were observed, one before and one after this series of complete ones. Copulation took place at the end of the first cycle only.

Histological examination of the ovaries revealed complete absence of follicles or tissue of obviously follicular origin. A typical section (Delafield's hæmatoxylin) of the left ovary is shown in Plate 3, fig. IIA. From this it will be seen that the mass of the ovary consists of cords of the first post-irradiation proliferation, in the midst of which are to be seen the cavities containing the remains of the follicles. Round the periphery a few cords, representing the beginning of the second proliferation, are to be seen budding off from the germinal epithelium. The germinal epithelium and the cords of the second proliferation stain up more deeply than the first proliferation tissue. The necrotic material in the cavities is barely perceptibly stained by hæmotoxylin. The uterus of this animal was perfectly typical of the normal di-œstrous uterus (autopsy was made 3 days after last œstrus). There can be little doubt, therefore, that this animal had an œstrous cycle in the complete absence of follicles.

M 6*a*.—On dissection 15 weeks after irradiation and after vaginal smearing for 54 days (18 weeks old), the broad ligament and mesenteries were loaded with fat, but the organs looked quite healthy. Only two periods of œstrus and two copulations (26 days apart) were observed in this animal, the last being three days before autopsy. Autopsy was performed at this time, so that if ovulation had occurred at the last period, *corpora lutea* should have been found after the *post-mortem*. This animal was peculiar in having a very restricted vulva, and the abnormality was so pronounced that not only was difficulty experienced in smearing the vaginal contents, but neither of the two vaginal plugs observed was evacuated. Both had to be removed with forceps after being in 3 and 2 days respectively. The ovaries were larger than usual in sterilised animals, and on microscopical examination it was found that besides the old follicle cavities there were, among the cords of the first proliferation, one or two structures appearing to consist of luteal tissue. No follicles or tissue of obviously follicular derivation were, however, present. Cords of the second proliferation were comparatively scarce, and but few had reached the tubular stage. The abnormal length of time between the two periods of œstrus remains anomalous, unless it should be connected with the presence of luteal tissue.

M 6b.—This animal had a very similar history. Killed 14 weeks after irradiation and vaginal smeared for 46 days, only two periods of œstrus were observed, and these were 33 days apart. They were, however, very definite periods of œstrus, accompanied by marked cornification of the vagina and also by coition. The mouse was killed immediately on the discovery of the vaginal plug, and dissection revealed the uterus in a typical state of œstrous distension. Unfortunately, the ligature put round the cervical ends of the cornua to preserve the inflation during removal and fixation cut the uterus. The organ immediately became deflated and no section is therefore available of the œstrous uterus.

Microscopically, both ovaries contained a large number of cavities within which were the necrotic remains of follicles. In both cases, also, the first proliferation was well marked and the cords of the second invasion were fairly pronounced, being intermediate in amount between M 1*d* (Plate 3, fig. IIA) and M 6*c* (Plate 3, fig. IIB). The left ovary was peculiar in containing a large cavity which, inside its very definite fibrous capsule, appeared to contain no tissue whatever ; certainly no ovum or follicular cells were present.

M 6c is a remarkably good case. It was put on vaginal smearing 7 weeks after irradiation and was only examined for 26 days. During this time, however, 3 complete cycles and four copulations were observed. These three cycles have an average duration of a little over 7 days. The pro-œstrous symptoms were perfectly normal, and finally when the *post-mortem* examination was made immediately after the last copulation, the uterus was found to present a typical œstrous condition. A view of this inflated uterus *in situ* is shown in Plate 5, fig. IVC. This is a perfectly typical appearance for an œstrous uterus. The ovary, figured in Plate 3, fig. IIB, shows complete absence of immature, mature or ruptured follicles. The whole ovary is very similar to that of M 1*d*, except that the darkly staining second proliferation is remarkably pronounced. This animal is particularly interesting in showing that a perfect œstrous cycle can occur in the absence of any ova or follicular tissue, and will even occur in the presence of very appreciable amounts of tubular tissue.

M 6e, although observed for 85 days, was not found to have an œstrous cycle, and, so far as could be ascertained, copulation did not take place. When dissected, at nearly 5 months old, the ovaries were found to be very small, one, in fact, being scarcely visible. In addition, the uterus was seen to be very thin and apparently atrophic. When the ovaries were examined microscopically a most peculiar condition was found. The greater part of the organs was occupied by tubules, and these, pushing in from the periphery, had squeezed the old ovarian tissue to the centre. No signs of follicles or oocytes were to be seen. On sectioning, the uterus was found to be clearly retrogressive. If the tubular tissue in the ovary had been present for any length of time, to the extent found on dissection, the absence of œstrous periods and the atrophy of the uterus is adequately explained, and this case cannot be said to be definitely opposed to the other results described.

M 6*f* was put under observation at 3 months old and was observed daily for 42 days. During this time three periods of œstrus, each accompanied by copulation, were observed. The two complete cycles observed occupied 16 and 17 days respectively. On dissection, the ovaries were seen to be of fair size, but, examined microscopically, oocytes, follicles or tissue of obviously follicular origin were found to be completely absent. The cavities containing débris were comparatively few, as were tubules of the second post-irradiation invasion.

M 7*a and c*.—These two females have an almost identical history and may be dealt with together. Daily observation of both was begun when the animals were 3 months old, and *a* copulated on 28th day of observation, while *c* copulated on the 11th. Subsequently to these dates, however, neither animal showed any signs of coming into œstrus and the autopsies were made on the 85th day of observation. Both showed almost identical microscopical and macroscopical conditions. Neither follicles nor oocytes were present, and the entire ovary appeared to consist of tissue of the first invasion. Cords of the secondary growth were almost entirely missing. Fatty degeneration was apparent in all the four ovaries. These two animals tend to stand apart from the rest of the series, but it is justifiable to conclude that both experienced œstrus in the absence of follicles or oocytes.

M 8.—This female was put under observation at 9 weeks old, and daily examinations were made for 30 days. During this time two periods of œstrus, 13 days apart, were experienced, each one being preceded by the normal vaginal symptoms. On dissection, the uterus appeared to be quite normal, and the ovaries were of fair size. Microscopically, the latter were found to contain no follicles or oocytes. Few cavities were observed, and the tubules of the second invasion were not pronounced.

M 9 had an almost identical history. Thirty daily observations were made and two periods of œstrus, 16 days apart, were observed. Both periods were preceded by the normal symptoms. On autopsy, macroscopical, and microscopical conditions entirely similar to *M* 8 were found. (Plate 3, fig. IIc.)

M 10 was also examined for 30 days, during which time three œstrous periods embracing two complete cycles, were found. Copulation took place on each occasion. The duration of the cycles was respectively 11 days and 12 days. The ovaries were quite devoid of oocytes and follicles, and were entirely comparable with those of the two preceding cases.

M 11 was put on to vaginal smear examination at 11 weeks old. Copulation was observed to have taken place on the second day of observation. This, however, was not repeated, and on the 28th day double ovariotomy was performed. Sectioning of the ovaries revealed a condition of complete sterilisation, both ovaries consisting almost exclusively of tissue of post-irradiation growth.

M 12 was examined for 17 days only, but during this time two œstrous periods, preceded by the normal symptoms, were observed. The one complete cycle had a duration of 15 days. The animal was killed immediately after the detection of the second vaginal plug, and on dissection the uterus was found to be in the inflated condition characteristic of œstrus. A section of this uterus is shown in Plate 4, fig. IIIE. The ovaries were found to show the typical sterilised condition.

M 13 was examined for 21 days before double ovariotomy was performed. During this time two œstrous periods, 18 days apart, were observed. Complete absence of follicles and oocytes was found when the ovaries were examined. The vaginal examinations were continued for 7 weeks after the double ovariotomy, but no further œstrous periods were detected.

M 14 was examined for 26 days without any œstrous period occurring, and, since it thus appeared that no cycle would be found, the possibility arose that this animal might show abnormally advanced development of tubules, as in the case of M 6*e*. In accordance with this supposition, it was reserved for further experimental work. In any case it is reasonable to suppose that this animal, in keeping with the others of the series, was completely sterilised.

M 17 was examined for 35 days before double ovariotomy was performed. During this time four œstrous periods, each accompanied by copulation, were observed. The cycles were respectively of 6, 12 and 14 days duration. After excision the ovaries were found to exhibit the usual irradiated condition, except that a considerable number of the tubules were present.

M 18 was examined for 25 days only; at the end of this period the animal became ill and was killed. Three œstrous periods, including two complete cycles, were, however, observed, but no copulation took place. Each cycle extended over 11 days. On dissection, the ovaries were found to be very small; the right ovary, in fact, was only found after a prolonged search. The uterus, however, was fairly well developed. Microscopically, the ovaries were both found to be quite devoid of ova and follicle tissue.

M 20 was examined for 35 days before double ovariotomy was performed. During this period only two œstrous periods were observed, but only the second was accompanied by copulation. In each case, however, intense cornification was observed for several days. The ovaries were found to be quite devoid of oocytes and follicles, but they were peculiar in that the tissue of the first post-irradiation invasion was very tubular in structure and showed many mitoses. The vaginal examinations were continued for 6 weeks after the ovariotomy, but no further symptoms of œstrus were observed.

M 21 was examined for 28 days before double ovariotomy and for 40 days afterwards. Before the removal of the ovaries two œstrous periods, 18 days apart, were observed. Copulation, however, only occurred once. When removed the ovaries were found to contain no oocytes, follicles or *corpora lutea*, and the follicle cavities were very scarce. The tissue of the first invasion was less tubular than M 20. Subsequent to ovariotomy, no periods of œstrus were observed.

M 22 was under observation for 36 days before the ovaries were removed. During this time 3 periods of œstrus were observed, the intervals being respectively 18 days and 11 days. After removal, the ovaries were found to show the typical sterile state, except that follicle cavities were practically non-existent and the thickening of the germinal epithelium for the second post-irradiation proliferation was well marked. After ovariotomy, the animal was observed for 48 days without any signs of œstrus appearing again.

M 23 had 42 daily vaginal smear examinations made before the ovaries were removed. Two periods of œstrus, 16 days apart, were observed, but in neither case did copulation occur. The ovaries were found, as in the previous cases, to be quite sterile, no oocytes, *corpora lutea*, or follicles being present. The follicle cavities were, however, fairly numerous. The tissue of the first ingrowth was very tubular, and similar to M 20, except that there was not the abundance of mitoses. The second proliferation was not pronounced.

M 24 was observed for 40 days without any appearance of œstrus, and as it appeared that this animal might be a case similar to M 6*e*, with an abnormally advanced development of tubes, the animal was kept for further experimental work. It is to be supposed, however, that it agrees with the others of the series in being sterile.

M 25 was under examination for 30 days before ovariotomy, during most of which time it was in bad condition, and had a tumour on the neck. In spite of this, however, four œstrous periods were observed, though no copulation took place. The three complete cycles observed were respectively 8, 8 and 6 days in length. Microscopical examination of the ovaries showed a condition almost identical with that found in the ovaries M 20, the animal being, of course, quite sterile. After ovariotomy, no signs of œstrus were observed during the 52 days that vaginal smearing was continued.

M 26 was killed after only 22 days' examination, owing to having a bad attack of diarrhœa. Three periods of œstrus were, however, observed, copulation occurring once. The two cycles were 6 days and 5 days in length respectively. The ovaries contained no oocytes, follicles or *corpora lutea*, and were in general very similar to those of M 25.

Table 1.—Summary of Œstrous Histories of Sterilised Mice.

No. of female.	Days under observation.	No. of œstrous periods observed.	No. of vaginal plugs.	Number of complete cycles.	Length of complete cycles (days).	Average length (days).
M 1*d*	37	4	1	3	5–13–12	10
M 6*a*	54	2	2	1	26	26
M 6*b*	46	2	2	1	33	33
M 6*c*	26	4	4	3	7–8–7	7·3
M 6*e*	83	None	None	None	—	—
M 6*f*	42	3	3	2	17–16	16·5
M 7*a*	85	1	1	0	—	—
M 7*c*	85	1	1	0	—	—
M 8	30	2	2	1	13	13
M 9	30	2	2	1	16	16
M 10	30	3	3	2	11–12	11·5
M 11	28	1	1	—	—	—
M 12	17	2	2	1	16	16
M 13	21	2	2	1	18	18
M 14	26	None	None	None	—	—
M 17	35	4	4	3	6–12–14	10·7
M 18	25	3	0	2	11–11	11
M 20	35	2	1	1	13	13
M 21	28	2	1	1	18	18
M 22	36	3	2	2	18–11	14·5
M 23	42	2	0	1	16	16
M 24	40	None	None	None	—	—
M 25	30	4	0	3	8–8–6	7·3
M 26	22	3	1	2	6–5	5·5
M 27	47	1	1	0	—	—
Totals	—	53	36	31	—	12·7

M 27 was observed for 47 days before ovariotomy, but only one œstrous period was observed during this time. Copulation took place. After ovariotomy no further sign of œstrus was detected. The ovaries were devoid of oocytes, follicles and *corpora lutea* and consisted almost exclusively of tissue of the first post-irradiation proliferation, and the later proliferation was not well marked. Follicle cavities were plentiful.

Summary of sterilised animals.—The records given above of the œstrous history of the first 25 animals completely sterilised at weaning time is summarised in Table I above.

This gives an average of 14·7 days for cycles preceded by copulation, and one of 9·3 for cycles not preceded by copulation.

It should be emphasised that the cases described above are not selected animals. The account contains the complete details for the first 25 sterilised animals whose œstrous histories were studied.

VII. *Discussion.*

Statement of results.—Of the 25 completely sterilised animals dealt with in the preceding section 22 were observed to have at least one œstrous period, while one of the remaining three had a development of tubes in the ovaries which might very well be incompatible with the occurrence of œstrous phenomena, especially in view of the atrophy of the uterus. Fifty-three periods of œstrus were in all observed in these mice, 36 of which were attended by copulation. The 31 complete œstrous cycles recorded had an average length of 12·7 days, which cannot be considered abnormal. If these results are to be considered valid—and at the moment it seems difficult to believe otherwise—the occurrence of numerous and, in many cases, regular periods of œstrus in animals totally devoid of oocytes and Graafian follicles is demonstrated, and the theory of the Graafian follicle as the causative factor of œstrus needs to be reconsidered. In view of the importance of this conclusion, it is proposed to consider briefly the possible criticisms which could be levelled against the experiments and the conclusions drawn from them, and the steps which have been taken to answer such criticisms in advance.

Possible objections to the experiments and conclusions.—In the first place, though it is obvious that the ovum and the organised structure of the follicle are destroyed, there was the possibility that the mass of tissue composing the irradiated ovary was follicular epithelium. To deal with this question, the development of the ovary following irradiation was worked out from animals killed at weekly intervals after irradiation. This material (which will be fully dealt with in the collaboration paper which is to be published shortly, and which is summarised in Section IV of this present paper) makes it quite evident that no tissue of follicular derivation is present in the vast majority of the irradiated ovaries, the only exception being the small spurious *corpora lutea* found in one or two cases. No difference can be detected in the œstrous periods of animals with these *corpora lutea* and those whose ovaries are devoid of them. Furthermore, it should be pointed out that in none of the ovaries of any of the 25 animals described are there any structures which have any appearance of being cyclic,* and therefore, whatever the exact nature of the tissue composing the ovary may be, that the cyclic liberation of the stimuli resulting in œstrus has to be accounted for.

Secondly, in view of Davenport's work, it seemed possible that normal ovarian tissue might have regenerated round some part of the capsule or elsewhere, and was not detected on dissection, and that this accounted for the occurrence of œstrus. If this had been so, it would have been expected that pregnancy would have resulted (the animals were all mated with normal males), but no pregnancy was ever detected in any of the 25 animals discussed in the previous section. It was clear, however, that this possibility could be experimentally proved or disproved, and to this end many of the experimental animals which had shown œstrus were subjected to double ovariotomy. The immediate cessation of cyclic activity in all cases showed, beyond doubt, that the sterile ovaries, which were excised, examined, and found to be devoid of follicles, included the actual tissue responsible for the production of œstrus.

Lastly, are the alleged periods of œstrus above suspicion ? As regards the actual detection of œstrus the following points may be made :—

(*a*) As regard the vaginal smear technique, the changes taking place in the sterile females were precisely similar to those found in the normal animals of the same colony.

(*b*) Since mice, in common with other animals, will only copulate at œstrus, copulation is the ultimate criterion of œstrus, and 36 out of 53 œstrous periods observed in the sterile mice were attended by copulation. Failure to copulate was invariably correlated with bad condition of the animal (see below).

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* In Part III of this paper it will be shown that after irradiation of the adult true *corpora lutea* may persist in the sterilised ovary for months, and that such persistent *corpora* have no influence on the œstrous cycle, and may therefore be regarded as non-functional.

(*c*) In addition, where animals were dissected immediately on the detection of œstrus by the vaginal smears, the uterus always presented the inflated condition typical of œstrus in the normal mouse (see Plate 5, fig. IV).

There can be little doubt, therefore, that the œstrous periods in the sterile mice differ from the normal only in the absence of ovulation; that is to say, the uterine and vaginal cycles are intact, but no ovarian cycle is detectable. As regards the cycles, it should be emphasised that the œstrous phenomena observed in the sterile animals are clearly cyclic, there being no question of any condition of nymphomania. One last point remains to be considered. While many of the sterile animals had a series of œstrous periods at normal intervals, several cases occurred where the interval between two periods was abnormally long, or where only one isolated period was detected. Even with these animals, it is clear that they did have at least one œstrous period in the absence of oocytes and follicles, but it may be argued that normal cyclic activity was not present.

In assessing the weight of this criticism, two facts need to be remembered. Firstly, the small size of the animals when irradiated at 3 weeks old precluded the possibility of adequately protecting the body as a whole from the rays, and the result is that the whole animal is affected by the irradiations. As a consequence, the young are much retarded in general condition and growth, and often fail to become good specimens of mice.* A regular œstrous cycle is notoriously dependent on good condition, and it is not surprising that some of such animals should have erratic cycles. Certainly this factor would appear to account for the occasional failure to copulate at œstrus. (See Parkes and Drummond (22) for the effect of vitamin A deficiency on œstrus.) Secondly, the gross fatness of many of these sterile animals must be taken into account (see Plate 5, fig. V). Excessive fatness, especially of the viscera, is well known as an inhibitor of œstrus, and the fatty degeneration found in the ovaries of, for example, M 7*a* and *c* would, if occurring in the ovaries of a non-experimental animal, be considered an adequate explanation of the non-occurrence of a regular œstrous cycle.

It is difficult, therefore, to put forward criticisms which entirely invalidate the experiments described in this paper, and the conclusions drawn from them, and it is therefore necessary to suppose that, under certain conditions at any rate, perfectly normal œstrous activity of uterus and vagina, together with the usual psychic manifestations, may be exhibited in the complete absence of oocytes, follicles and *corpora lutea.* Implications of this conclusion will be considered in the next section.

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* We have yet to determine what damage, if any, is done to other essential organs by mass irradiation at this age with the doses employed for these experiments.

The mechanism of the œstrous cycle.—The results discussed above lead inevitably to the conclusion that in the mouse, at any rate, the phenomena of œstrus may occur quite independently of the presence of any follicles, and it is now necessary to consider the bearing of this conclusion on present views of the regulation of the œstrous cycle. In the first place, it may be of use to consider whether any experimental or deductive evidence from other sources or relating to other mammals is to be found. Chief in this connection are the experiments of Marshall and Runciman, quoted in Section I, where destruction of all the large follicles in bitches did not delay the onset of the next œstrus; also, there may be mentioned the supposed cases of menstruation without ovulation in Primates (Heape). In view, however, of the uncertainty about the exact nature of menstruation, this evidence is of doubtful relevance, and it is in any case mutually incompatible with facts to be adduced later. It would seem, therefore, that little outside support can be found for the present experiments.

In the sterilised animals the hormone responsible for the causation of œstrus is apparently being produced by inter-follicular elements, but does this mean that this is the normal site of origin ? Since it is highly improbable that a group of cells would take on secretory functions entirely alien to them, it must be supposed that in the normal animal the inter-follicular tissue at least takes a part in elaborating the œstrus-producing hormone. But it is definitely proved that the hormone can be extracted from follicles, and it would thus appear that the hormone is either produced both within and without the follicles, or else that it is produced outside the follicles, its presence inside being purely secondary. In the last resort, the fact that the hormone can be extracted from follicles is no evidence as to the site of production, because it has been shown by the present writer, working in conjunction with Mr. C. W. Bellerby (21), that the hormone can be extracted in even larger amounts from cow and pig ovaries from which all large follicles have been removed.

There is a further point, too, which bears on this aspect of the subject. Allen and Doisy themselves say that the hormone can be obtained from any pig follicle larger than 5 mm., which in the case of the pig is a quite immature one. Such immature follicles are present in the pig ovary during the greater part of adult life, and thus it is quite evident that the hormone is not restricted to maturing follicles, as is suggested by the view that the periodic maturation of follicles is sufficient to account for the coincident periodicity of œstrus. So far, then, we may sum up by saying that the œstrus-producing hormone in the mouse is produced by inter-follicular tissue, though possibly also by the follicles.

The next question is: Is this statement likely to be valid for other mammals? Evidence sufficient to answer this is lacking at the moment, but when the essential similarity of the reproductive processes in different mammals is considered, and taken in conjunction with the non-specific nature of the hormone itself, it would be not unreasonable to suppose that the site of origin of the hormone is essentially the same in other mammals.

The greatest difficulty in the way of accepting the supposition that the œstrous hormone is produced by the apparently non-cyclic inter-follicular tissue is to account for its periodic action. The fact noted by Dodds, Dickens and Wright (11), that a constant supply of the hormone will keep an animal constantly in heat, shows that its action must in the ordinary way be periodic, coinciding with pro-œstrum and œstrus, and it is difficult to see how the non-cyclic inter-follicular tissue can arrange for this periodicity. It is clear from the experiments recorded in this paper that the co-operation of the cyclic follicles or *corpora lutea* is not required. However, it was known before that the hormone was present in follicles of all ages, and also to some extent in the inter-follicular tissue (one estimation by Dodds, Dickens and Wright), so that the problem of periodic action (young follicles, for instance, being always present) is really not a new one.

There is, however, one point which makes the problem more difficult. To suppose that the maturing follicles were responsible for the appearance of œstrus was to explain easily the connection between the first œstrus and the first ovulation, and similarly the connection between the last œstrus and the last ovulation, œstrus and ovulation beginning synchronously at puberty and ending synchronously at the menopause. Now, however, there is the additional fact to explain that œstrus can start at puberty without any apparent change in the ovary. Since, however, it is necessary to credit the ovary with the capacity to produce œstrus periodically during adult life without any apparent change in itself, there is no real reason why it should not be credited with the capacity to bring about the first periodic action at puberty.

In conclusion, it may be said that the experiments recorded in this paper suggest that there exists in the mammalian ovary a periodicity which is responsible for the cyclic development of Graafian follicles and the coincident cyclic action of the hormone, causing the requisite hypertrophy of the uterus.

In the absence of follicles through irradiation or other destruction, the rhythmic action of the hormone proceeds in the normal way, but is unacccompanied by ovulation.

Clinical applications.—The conclusions reached above have a not unimportant bearing on the theory of the human menstrual cycle. The results of irradiation of the human ovary appear to have been somewhat contradictory, but most authors agree (see, for instance, Wernier (27)) that amenorrhœa is brought about temporarily, if not permanently, by adequate exposure.

The results recorded in the present paper suggest that irradiation does not inhibit the appearance of œstrus, and thus presumably œstrus should occur after X-ray sterilisation of the human subject. If this view is correct, menstruation, which is inhibited by irradiation, is not the physiological analogue of the œstrus period of the lower mammals, and is probably therefore of the nature of pseudo-pregnant degeneration. In this connection the recent work of Seitz and Wintz (25) is of considerable interest. These authors state that irradiation during the first half of the intermenstrual period inhibits the next menstruation, whereas exposure during the second half of the intermenstrual period has not this effect. In either case amenorrhœa sets in afterwards.

This work is most readily explained as follows. If ovulation is assumed to occur about the middle of the intermenstrual period, irradiation during the first half of this period, by destroying the maturing follicle, would prevent the formation of the *corpus luteum* necessary for the production of the premenstrual congestion of the uterus. The next menstrual period would therefore be inhibited. Irradiation during the second half of the intermenstrual period, on the other hand, would not prevent the formation of the *corpus luteum,* which, being immune to X-rays, would go through the usual cycle of growth and regression, menstruation appearing in the ordinary way. Seitz and Wintz have therefore added very considerably to the evidence which tends to show that human menstruation is a process of pseudo-pregnant degeneration, and not a pro-œstrous phenomenon.

Between the two lines of work very strong support is afforded to the pseudo-pregnancy theory of human menstruation. This view also explains Solomons' and Gatenby's (26) results on the administration of liquor folliculi and *corpus luteum* extracts in cases of amenorrhœa, the former proving useless, while the latter were efficacious. It would seem, therefore, that exposures to X-rays sufficient to sterilise the ovary completely would, by prohibiting the formation of *corpora lutea,* be of value in cases of dysmenorrhœa or menopause complications, where it was desired to produce the condition of amenorrhœa.

VIII. *Summary.*

(1) Of 25 mice sterilised by exposure to X-rays at 3 weeks old, 22 experienced at least one period of œstrus when puberty was reached. In all, 53 periods of œstrus were observed in these animals, many of which exhibited a series of complete cycles of the normal length.

(2) Since none of these animals had oocytes, follicles, or follicular tissue in the ovaries, it is concluded that the cyclic structures of the ovary are not essential for the occurrence of the normal cyclic activity of the uterus and vagina.

(3) Possible criticisms of these results are discussed, and it is concluded that the œstrus-producing hormone is elaborated by the inter-follicular tissue, though also possibly by the follicles under normal conditions.

(4) The problem of the cyclic action of the sterilised ovaries containing no apparent cyclic structures is dealt with, and it is concluded that the normal ovary possesses some cyclic mechanism which brings about the rhythmic production of œstrus and the coincident maturation of follicles.

I should like to take this opportunity of expressing my thanks to Prof. A. V. Hill, F.R.S., for his interest in the work, and also to Dr. F. H. A. Marshall, F.R.S., and Prof. J. P. Hill, F.R.S., for their advice and criticism.

Finally, it is a pleasure to acknowledge my debt to Mr. F. Melville, radiographer to the Department of Anatomy, without whose co-operation this work would have been impossible. All the irradiations recorded in this paper and a previous one (Parkes, 17) have been carried out by Mr. Melville.

The mouse colony from which the experimental animals were drawn was maintained during the period primarily with the aid of a grant from the Ministry of Agriculture and Fisheries, but also with the additional aid of a grant from the Government Grant Committee of the Royal Society. To both of these my thanks are due.

DESCRIPTION OF PLATES.

PLATE 3.

FIG. I.—A. Ovary of normal adult mouse showing mature follicles and *corpora lutea.* × 40.

B. Ovary of normal mouse at 3 weeks old, showing a large number of immature follicles. × 60.

FIG. II.—A. Ovary of M 1*d* at 10 weeks old, having been irradiated at 3 weeks old. No follicles are present, but the spaces are probably cavities left after the degeneration of the follicles. I.G. is the tissue produced immediately following irradiation, and E. is the thickened germinal epithelium which is budding off tubules (T). × 60.

B. Ovary of M 6*c*. Description as (A). × 60.

C. Ovary of M 9. In this case the thickening of the germinal epithelium and the production of tubules as the second phase of post-irradiation growth is not shown to any great extent. × 60.

PLATE 4.

FIG. III.—A. Normal adult uterus in diœstrus. M. Muscular layer, S. Stroma, G. Glands, E. Epithelium. × 30.

B. Uterus of M 2*b*, showing complete development after sterilisation. Lettering as in IIIA. In many of the sterilised mice the uterine epithelium and the outline of the lumen is very convoluted, as shown here. × 40.

C. Normal œstrous uterus. Greatly distended with fluid. M. Muscular layer, S. Stroma, Sp. Mass of spermatozoa and epithelium cells. × 25.

D. Œstrous uterus of M 1*b*, showing condition identical with normal œstrous uterus. Lettering as C. × 25.

E. Œstrous uterus of M 12. In this case the uterus had partly deflated before fixing, but the dilated condition can be seen. Lettering as C. × 25.

PLATE 5.

FIG. IV.—A. Dissection of normal diœstrous uterus, showing thin uterus. U. Uterus, O. Ovaries, B. Bladder.

B. Dissection of normal œstrous uterus showing dilation. Lettering as A.

C. Dissection of œstrous uterus of M 6*c*, showing typical œstrus appearance. Lettering as A.

D. Dissection of œstrous uterus of M 12, showing typical œstrus condition. Lettering as A.

FIG. V.—M 6*a*, showing abnormal adiposity. × $\frac{2}{3}$.

(Photomicrographs by Mr. F. J. Pittock.)

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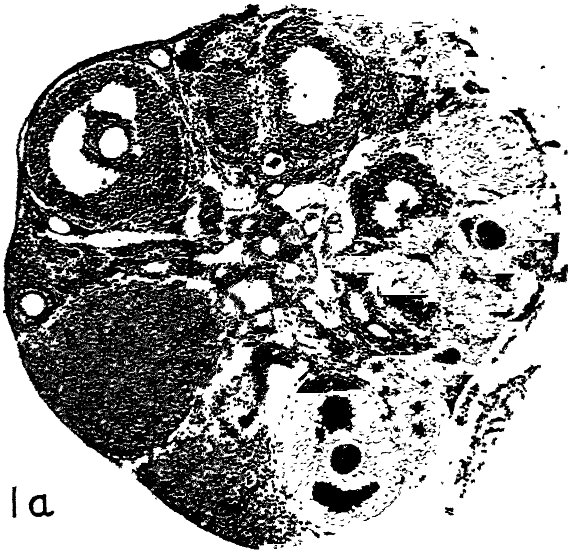

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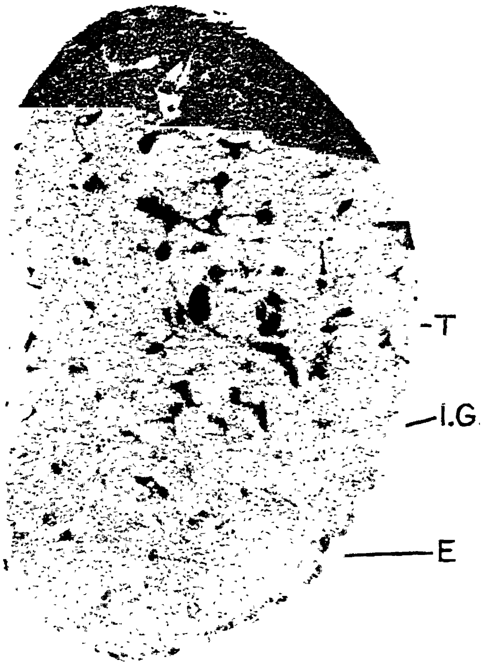

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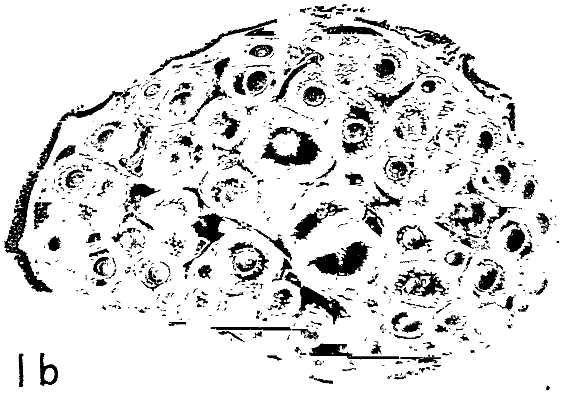

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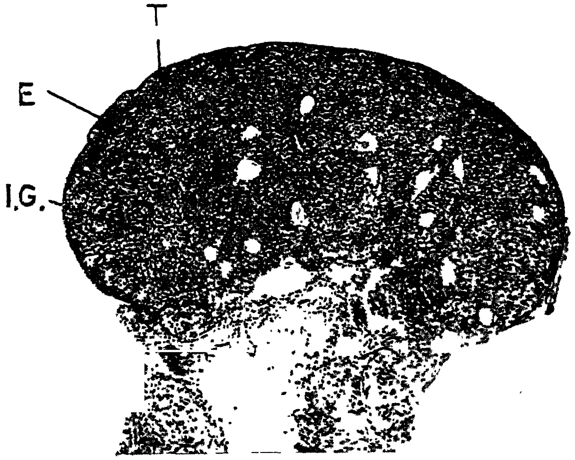

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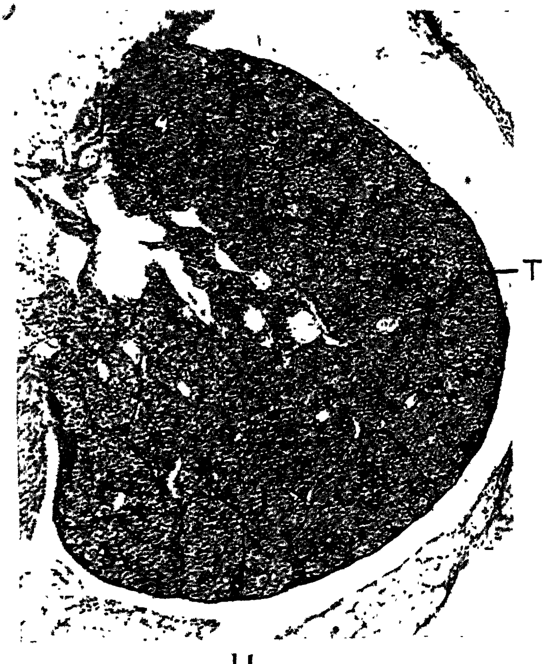

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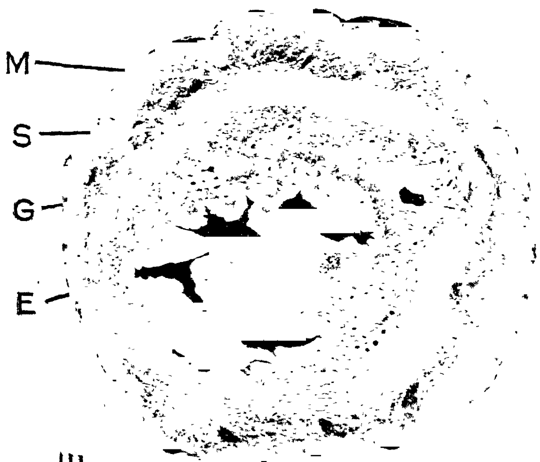

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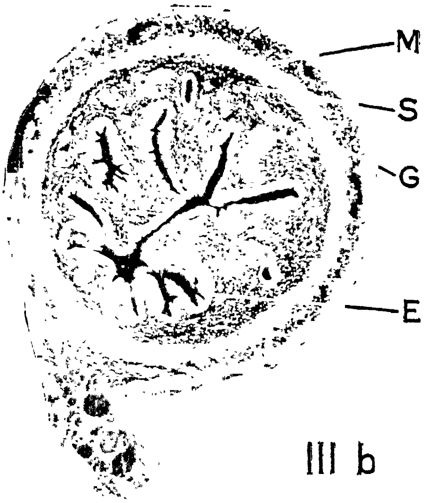

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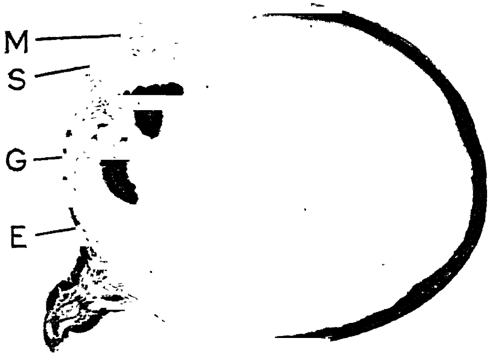

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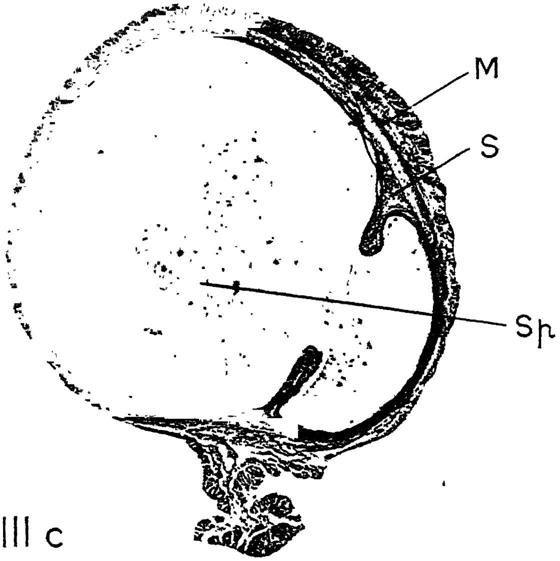

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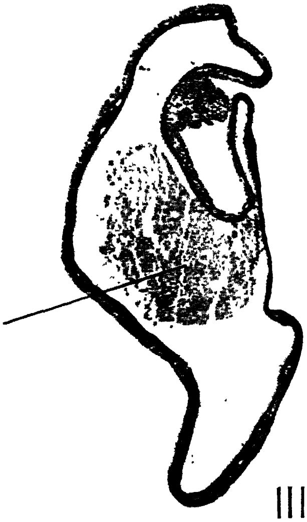

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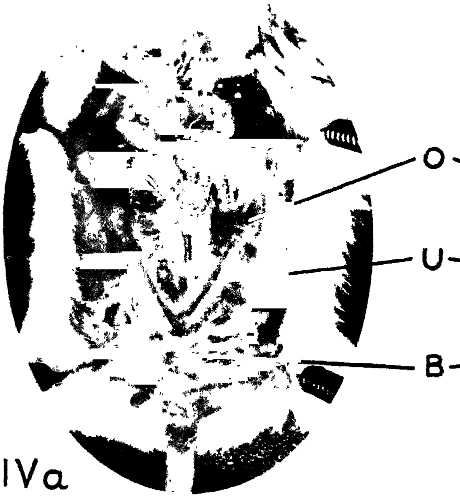

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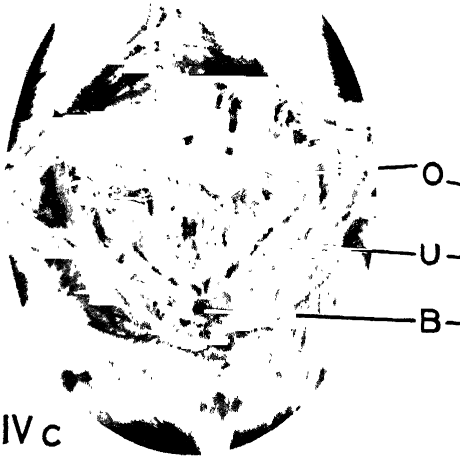

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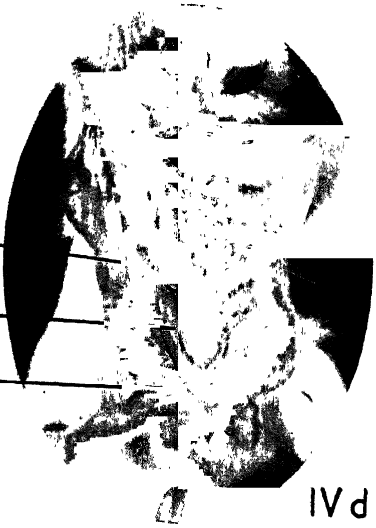

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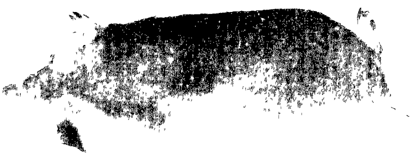

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The Equations Applicable to Simple Hæmolytic Reactions.

By Eric Ponder.

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The purpose of this paper is to develop and to consider in detail some of the expressions which may be applied to reactions involving simple hæmolytic systems. In doing so, a number of rather unusual conditions have to be considered; these may first be set down.

1. It has been shown (1) that the curves obtained by plotting the amount of hæmolysis, expressed as a percentage of the total, against time, are sigmoid in form, the sigmoid curve often presenting a considerable degree of skewness in one or the other direction. In explanation of this form, it was suggested that the cells of the system present different degrees of resistance to the action of the lysin, these resistances being distributed according to a symmetrical

frequency curve, and that the skewness encountered is due to the presence of secondary reactions, principally the reaction of the liberated cell-contents with the lysin. Such suggestions are, in general, in agreement with the findings of Brooks (2), of Fulmer and Buchanan (3), and of other workers on hæmolysis and the allied problem of bacteriolysis. To the two papers mentioned the reader is referred for excellent discussions of both facts and elementary theory, as well as for reviews of the literature. It appears that the bulk of the recent investigation has shown that these variations in cell resistance, and the distribution of these resistances, are of fundamental importance in problems of this kind, and that it is completely fallacious to deal with such problems without allowing for the heterogeneity of the material involved.

As there is no evidence to the contrary, we shall provisionally take it that the distribution is of the type $n = Ae^{-b^2x^2}$, the resistances, in terms of the quantity of cell component transformed by the action of the lysin, being taken to be distributed according to an ideal frequency curve. It is of fundamental importance to observe that the abscissa of this curve is in units of cell component transformed, and not in units of time. Misunderstanding on this point is easy, from the fact that the resistances are customarily measured in the latter units.

2. We know that the process which we term hæmolysis is only the initial portion of a long reaction, which is continued after the completion of lysis as a stromatolysis of the ghosts. The evidence on this obvious point is sufficiently conclusive (4). The equations applicable to hæmolysis must therefore be derived from the consideration of the more prolonged reaction, and the end-point of complete hæmolysis must not be taken as a point beyond which no further reaction occurs.

3. The available evidence points to saponin, the bile salts, and lysins like them, forming compounds with the proteins of the cell envelope, and we therefore propose to take it that the lysin forms, with the particular cell component involved, a compound in many respects like an absorption compound. That lysis is due to reaction of this nature has been our expressed opinion throughout this series of papers, although we are aware that it is often stated that these lysins act as lipoid solvents. The evidence for the assumed combination with protein will be found in previous communications, and is supported by the findings of Oliver and Barnard (5), which indicate that the outer surface of the cell envelope contains as a principal component, not lipoid, but protein. There is no satisfactory evidence for the supposed solvent action of these lysins, and a consideration of the kinetics of the reactions would alone be enough to dispose of such a view.

4. In a hæmolytic system in which red cells and lysin are interacting, we have a very different state of affairs from that which occurs in a true solution. The difference lies in the fact that the reaction cannot proceed equally at all points in the fluid, for of necessity it is confined to the region of the cell surfaces, and these cells are separated from one another by comparatively great distances. A molecule of lysin, for example, situated midway between two adjacent but considerably separated cells, cannot react upon either of them. This condition is further encouraged by the fact that saponin, the bile salts, and many of the simple hæmolysins, tend to collect at surfaces to form concentrated layers. It is obviously incorrect to apply to a system in which such discontinuities of reaction occur the same treatment as is applicable to systems in which the reacting material is evenly distributed. This difficulty does not appear to have occurred to previous workers on the subject, who have used simple equations for chemical reactions to describe hæmolysis, and whose equations accordingly fail when applied to some of the most important phenomena.

5. It has been pointed out already that accompanying the principal lytic reaction there may be secondary reactions, due to the fact that the liberated cell contents react in some cases with the lysin, producing either an acceleration or an inhibition (1). The influence of these secondary reactions on the principal reaction constitutes a very difficult part of the problem.

6. It is necessary that the equations derived for the description of hæmolytic phenomena shall do more than describe merely the variation in time for lysis with concentration of lysin, or the variation of percentage hæmolysis with time. They must explain, from first principles, the time-dilution curves, the percentage hæmolysis curves, the relation of different hæmolytic systems to one another (6), and the curious results obtained by varying the number of cells to be hæmolysed (7) before they can be considered adequate. A number of theories have been hitherto advanced which deal with one or another of these points, but none which treats of them all.

We shall now develop the necessary equations.

Consider the nature of the reaction at those points in the system where it can occur—that is, in the neighbourhood of the red-cell surfaces. The equation for the formation of an adsorption compound, when deprived of those terms which have no meaning in our problem, is the well-known expression

$$\frac{dx}{dt} = \kappa(c - x)\,\mathrm{S}$$

in which κ is a velocity constant, S the surface at which formation takes place,

x the amount of reacting substance, in this case lysin, which is used up in the formation of the compound, and c the initial concentration of the reacting substance.

To utilise an expression of this sort as a basis for an equation relating to hæmolysis, it is to be remembered that the surface of formation is not constant. As lysis begins, it is S, the surface of the cells, but as lysis proceeds, liberated cell contents may form a new and ever-increasing surface of formation, provided that these liberated contents react with the hæmolysin. We shall take, first, the simple case in which the lysin reacts with the liberated cell contents, and is rendered inert, as in the case of saponin.

Suppose we have a population of cells of various resistances, and that these resistances are normally distributed according to $n = \mathrm{A}e^{-b^2x^2}$, n being a number of cells, and x the amount of lysin which requires to be transformed in a reaction with the cell surface in order to effect lysis. Then as hæmolysis proceeds, the number of cells hæmolysed will be $n' = \int_0^x \mathrm{A}e^{-b^2x^2}\,dx$. In these expressions the values of x in the indices are referred to the mean of the curve, while the limits of the integral are absolute values of x.

This integral must be replaced by another expression, for, used as it stands, it leads to insoluble equations. An approximation of sufficient accuracy is $n' = k'x$, the sigmoid curve which represents the integral of the distribution curve being thus replaced by a straight line through its extremities. (Fig. 1.)

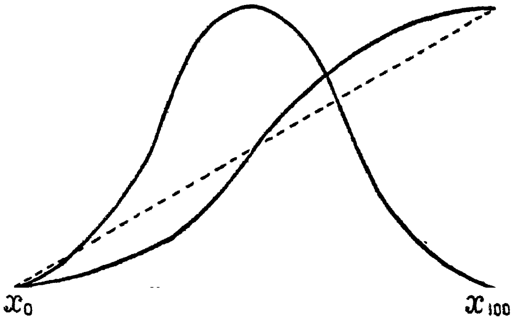

Fig. 1.

The liberated cell contents are clearly proportional to n', the cells hæmolysed, and so we have the added surface of formation as $\mathrm{S}' = kx$. It should be noted that k is not necessarily the same as k', for a unit of cell surface S may not be so effective in reacting with lysin as a unit of the new surface S′.

Now the lysin may react with either S or with S′. In the former case it tends to produce lysis, in the latter case it is rendered inert and has no effect on the cells. If x is the amount reacting with S and y the amount reacting with S′, we have $x/y = \mathrm{S}/\mathrm{S}'$, whence $y = kx^2/\mathrm{S}$. The free lysin at any moment is accordingly $(c - x - kx^2/\mathrm{S})$.

Further, as the lysin may react with either S or S′, the probability of its reacting with S is $\mathrm{S}/(\mathrm{S} + \mathrm{S}')$, or $\mathrm{S}/(\mathrm{S} + kx)$. Thus the lysin transformed by a reaction with the cells is

$$\frac{dx}{dt} = \kappa\,(c - x - kx^2/\mathrm{S})\,\frac{\mathrm{S}^2}{\mathrm{S} + kx}.$$

A rather tedious integration gives

$$\kappa t = \frac{1}{2\mathrm{S}\sqrt{(1 + 4kc/\mathrm{S})}}\log\frac{x[1 - \sqrt{(1 + 4kc/\mathrm{S})}] - 2c}{x[1 + \sqrt{(1 + 4kc/\mathrm{S})}] - 2c} + \frac{1}{2\mathrm{S}}\log\frac{c}{c - x - kx^2/\mathrm{S}} \qquad (1)$$

It is to be emphasised that in this equation x is not the total amount of lysin used up, but the amount used up in the reaction with the cell surface only. This is what is required, as t, the time for complete or percentage hæmolysis, refers to hæmolysis only, and not to accompanying reactions. It is also to be observed that the equation is not limited to the hæmolytic reaction proper, for, by considering values of x, the lysin used up in reacting with the cells, greater than that value which is sufficient to give complete lysis, we have an expression applicable to the succeeding stromatolysis. During the stromatolysis there is, of course, no further increase of the surface of formation, for the values of x which make the equation applicable to stromatolysis are greater than that which forms the limit of the frequency curve of cell resistances. This upper limit disappears in the making of the linear approximation $n' = k'x$, but must be remembered nevertheless. The lower limit is $x = 0$, although this corresponds to some negative value of x in the expression for the frequency distribution, which is, as is usual, referred to its mean as origin.

When $k = 0$—that is, when the liberated cell contents do not react with the lysis, as is probably the case when such a substance as sodium hydrate is used as a lysin—(1) becomes

$$\kappa t = \frac{1}{\mathrm{S}}\log\frac{c}{c - x} \qquad (2)$$

This is similar to the monomolecular expression advocated by Arrhenius and his pupils (8),

$$\kappa t = \log\frac{c}{c - x} \qquad (3)$$

which is well known in connection with chemical reactions of the first order. Unfortunately, if the curve represented by (3) be plotted, it lies throughout a large part of its course very close to the rectangular hyperbola

$$ct = \text{const.} \tag{4}$$

We say unfortunately, for Arrhenius and some of his pupils have used (4) as if it were something other than a very poor and extremely misleading approximation, and have obtained some totally inaccurate results as a consequence. The nature of the error involved can be seen at once by substituting $\delta = 1/c$ in (4); if that expression were true, the time-dilution curves of experiment would be straight lines instead of curves concave to the t-axis. Apart from this, the expression (3) used by these writers does not contain a term relating to surface; we shall see that this is a serious omission.

In the case of lysis by saponin and hæmolysins of its class, we have taken it that the liberated cell contents react with the lysin to render it inert. In the case of lysins such as sodium hydrate, it has been assumed that no reaction takes place with the liberated contents. There remains the case of lysis by sodium taurocholate and by the soaps, in which it appears that, as lysis proceeds, the reaction becomes more rapid. The acceleration of the reaction we have attributed to an effect exerted on the hæmolytic system by the liberated cell contents, and it remains to be considered how this is brought about.

There are three components in the system: (*a*) the lysin, (*b*) the particular cell component which is attacked, and (*c*) the cell contents; it is certain that (*a*) reacts with (*b*). The increase in velocity of the reaction as lysis proceeds might be explained either by the assumption that the cell contents act as a lysin, and that thus, as lysis proceeds, the amount of lysin in the system becomes increased, or that the lysin reacts with (*c*), the result of the reaction being that the lysin is increased in activity. This first assumption cannot be upheld, for we know that the liberated cell contents have not the properties of a hæmolysin. The second assumption leads to an expression which fails to account for the form of the percentage hæmolysis curve for the soaps and for sodium taurocholate, and in addition to this there is the objection that there is evidence that any reaction between the liberated contents and the uncombined lysin results in an inactivation of the latter rather than in an increase in its lytic power (9). We have therefore to assume that there is no secondary reaction between uncombined lysin and liberated cell contents, unless one resulting in inactivation and covered by an expression of the form of (1). At the same time, we explain the acceleration in the reaction as lysis proceeds to the liberated cell contents reacting with the cell component, probably after it is combined with a small amount of lysin as a result of the early stages of the reaction, and producing an altered distribution of the cell resistances. Such an assumption would not affect the applicability of (1) or of (2), for in the derivation of the second of these expressions no particular distribution of resistance was assumed, while the approximation used in the derivation of the first would certainly suffice even if the distribution were somewhat altered.

We might therefore expect that the time-dilution curves for the action of such lysins as sodium taurocholate and the soaps would be expressed by (2), for we shall show that, so far as time-dilution curves are concerned, (1) and (2) are indistinguishable experimentally, and that the explanation of the percentage hæmolysis curves would call for the introduction of new conditions regarding the distribution of the cell resistances.

In (1) and (2), which are the fundamental expressions to be considered, there are four possible variables, t, c, x, and S. If t and c are variables, the curve given is a time-concentration curve, related to the time-dilution curve of experiment. If t and x are variables, the resulting curve is the basis of the percentage hæmolysis curve. If we treat x and S as variables, we get relations which determine the behaviour of the lysin to different strengths of cell suspension and which should agree with the findings of (7). These expressions should also contain the explanation of the findings of (6) which concern the relation of different hæmolytic systems to one another. Each case may now be dealt with in detail.

1. *The Time-concentration and Time-dilution Curves.*

In (1) when $c = x + kx^2/\mathrm{S}$, t becomes infinite; the curve therefore approaches an asymptote determined by the value of $(x + kx^2/\mathrm{S})$. We take it as fundamental that the lysis of a certain number of cells corresponds to the using up of a certain amount of lysin; since x is the amount of lysin used up by the cells, it follows that x must be constant for all concentrations when 100 per cent. hæmolysis is complete. If, therefore, we plot the time for complete hæmolysis against c, the concentration of lysin added to bring about lysis, we should get a curve described by (1) in which we have κ, k, S, and x to determine. This curve will become the time-dilution curve of experiment by putting $2/c = \delta$.

Unfortunately, there is no way of determining these four constants except by trial and error; this involves a long series of computations, at the end of which one is by no means sure that one has selected the best values. The constant S may be at once put equal to unity, but the difficulty arises from the fact that the value of the asymptote, being determined by $(x + kx^2)$, is given by an unlimited number of values of x and k. One has to put k successively equal to 10, 20, 30, etc., find the value for x which will give the proper position for the asymptote as obtained by experiment, and then compute, for each value of k, the value of t in (1), the velocity constant being regarded as unity. From the curves so obtained the one which fits the experimental results best has to be selected, after various values have been given to κ. This process is laborious and unsatisfactory, for quite large variations in k alter the form of the curves but little, provided that the value of $(x + kx^2)$ be kept constant.

In order to illustrate this point we show in the following table values for t calculated from (1) for different values of k. All the curves approach the same asymptote, given by $(x + kx^2) = 0{\cdot}028$, a very common value in experiments on saponin hæmolysis at 25° C., and in each S and κ are taken as unity. The most usual value for κ in experiments with saponin at 25° C. is about 0·25, so the values in the table may be multiplied by four to give the approximate values of t which might occur in practice.

c.	$k = 0$.	$k = 20$.	$k = 40$.	$k = 60$.
0·20	0·148	0·120	0·118	0·113
0·10	0·329	0·279	0·261	0·249
0·05	0·819	0·713	0·640	0·624
0·04	1·194	1·002	0·912	0·885
0·033	1·841	1·525	1·422	1·347
0·02858	3·892	3·169	2·940	2·754

From the values of the table it will be seen that the effect of varying k is not a great one. The principal difficulty is, however, that it can be made to appear very much less, for in these calculations κ has been put equal to unity. If different values of κ be assigned to the curves they can be made to agree more closely; thus, if in the curve with $k = 0$ we put $\kappa = 1{\cdot}31$ we get values very close to those of the curve with $k = 60$ and $\kappa = 1{\cdot}00$, for the values are :—

$c =$	0·2.	0·1.	0·05.	0·04	0·033.	0·02858.
$k = 0$ .... $\kappa = 1{\cdot}31$	0·113	0·251	0·627	0·909	1·406	2·979
$k = 60$ .... $\kappa = 1{\cdot}00$	0·113	0·249	0·624	0·885	1·347	2·754

In an actual experiment it would not be possible to distinguish between any two of these values, except the last pair, 2·979 and 2·754. If, as is quite usual, the values of t were about four times those shown in the table, we might have the value 11·9 corresponding to $\kappa = 0 \cdot 33$, $k = 0$, and the value 11·0 corresponding to $\kappa = 0 \cdot 25$, $k = 60$. It would be barely possible to distinguish experimentally between two such values of t; values smaller than these would be certainly indistinguishable. Further, we have no independent means of finding the true value of κ; it is therefore impossible to determine, from the time-dilution curves alone, the proper values of k by fitting experimental and calculated results.

These considerations lead to the conclusion that equation (1) must be abandoned for practical purposes, so far as the time-dilution curves of experiment are concerned. We have sufficient accuracy in the use of equation (2), which is an approximation, and the application of which will give the form of the time-dilution curves satisfactorily, although it will give incorrect values of κ, the velocity constant.

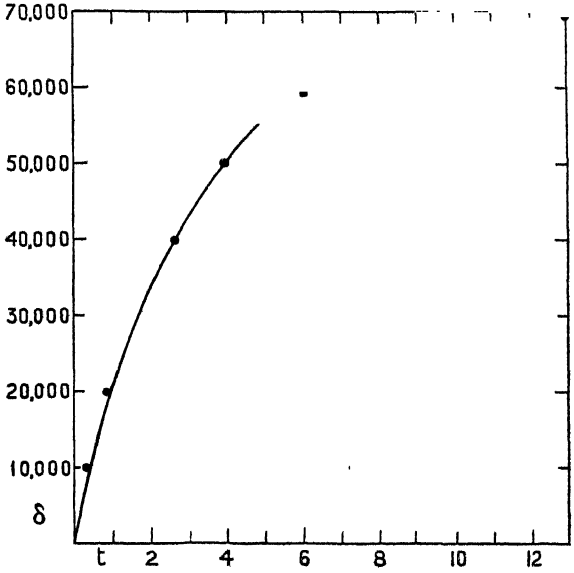

Fig. 2.

In fig. 2 is shown the correspondence between a typical experimental time-dilution curve for saponin acting at 25° C., and the values calculated for equation (2). The concentrations are converted into dilutions in the usual way, and the calculated results based on the values of $x = 0 \cdot 028$, $\kappa = 0 \cdot 303$. The fit is obviously very good; the only defect is a slight excess of the calculated values over the experimental values or high concentrations. Although these differences are smaller than those considered by most observers to be negligible, we are inclined to think that their magnitude indicates that some small factor has been omitted from the equations. The differences do not disappear on the substitution of the theoretically more correct equation (1) for (2), and we are satisfied that they cannot be accounted for by experimental error. Apart from this, the calculated values agree with the observed ones very closely.

Fig. 3 shows a time-dilution curve for sodium taurocholate, calculated from $x = 0{\cdot}33$, $\kappa = 0{\cdot}178$, and equation (2), which, as has been shown, is indistinguishable from (1). Here again the fit is excellent. The particular experimental curve used in this example is taken from (9).

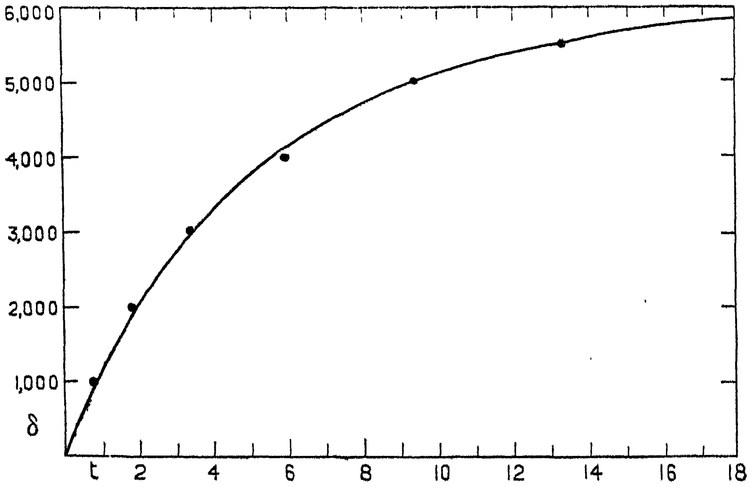

Fig. 3.

Fig. 4 shows the calculated curve and experimental results for sodium stearate acting as a lysin. The fit is satisfactory, thus showing that (2) is applicable. For the calculated curve κ was taken as $0{\cdot}222$. It may be observed that this lysin gives bad experimental end-points.

2. *The Percentage Hæmolysis Curves.*

The percentage hæmolysis curves give the relation of n', the number of cells hæmolysed, to t, the time, for various concentrations of lysin; n' is usually expressed as a percentage of the total number of cells. We have to derive these curves from the relations

$$n' = f(x) = \int_0^x \mathrm{A}e^{-b^2x^2}\,dx,$$

in which the limits of the integral are absolute values of x, the lysin used up, while the value of x in the index is referred to the mean of the frequency curve which is integrated, and

$$x = f(t)$$

which is given by making x and t variables in equations (1) or (2).

In (1) we can put $S = 1$, and calculate, for any known value of c, the initial concentration of lysin, the value of x, the lysin used up at various stages from the commencement to the completion of hæmolysis, corresponding to values of t. The value of t corresponding to complete hæmolysis is known as the result of experiment, and the value of x corresponding to this value of t is also known, for it may be obtained, interlocked with k, from the asymptote of the time dilution curve for the lysin acting on the same cell suspension. In like manner values of x corresponding to values of t may be calculated from (2). The curves obtained are exactly like those for reactions of the first order.

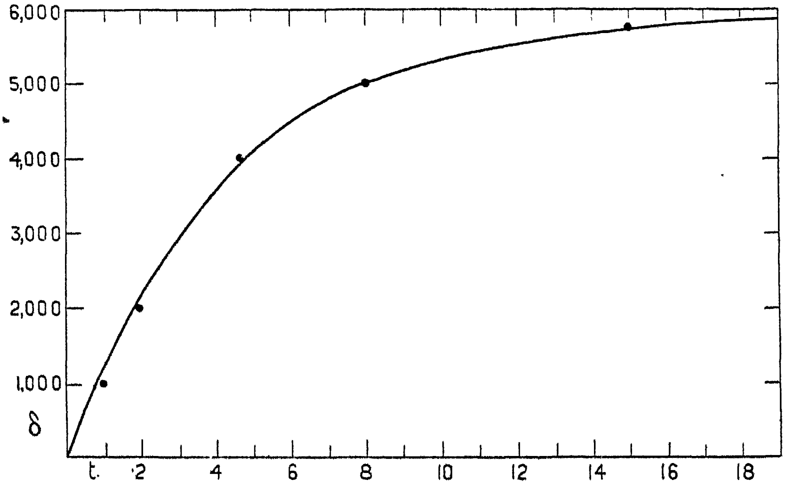

Fig. 4.

A reaction described by these curves proceeds through a population of cells, which, in the case of hæmolysis by saponin, acids, alkalies and lysins like them, is distributed according to a normal frequency curve. We omit for the time being the case of such lysins as sodium taurocholate and the soaps, to which we shall assume that the resistance of the cells is not symmetrically distributed. We have then to find the form of the percentage hæmolysis curve obtained by combining $n' = f(x)$ and $x = f(t)$; the complete analysis of the form is both difficult and unnecessary, and so it will be enough to deal with the most outstanding property of the resulting curve.

If the distribution of the population of cells is a symmetrical one, it will be plain that when half the lysin used up for complete hæmolysis is transformed half the cells will be hæmolysed, and so if in an experimental percentage hæmolysis curve we measure t_{50}, the time for lysis of 50 per cent. of the cells, and t_{100}, the time for complete hæmolysis, we shall have the ratio

$$\frac{t_{100}}{t_{50}} = \frac{\log \dfrac{c}{c - x}}{\log \dfrac{c}{c - \frac{1}{2}x}} \tag{5}$$

when the reaction proceeds according to (2). This expression gives a simple relation between the skewness of the percentage hæmolysis curve and the underlying reaction between the lysin and the cells, the measure of skewness being the ratio of the distance of the median from the extremes of the curve. If, on the other hand, the reaction between the cells and the lysin proceeds according to (1) we have

$$\frac{t_{100}}{t_{50}} = \frac{\dfrac{1}{2\sqrt{q}} \log \dfrac{x(1 - \sqrt{q}) - 2c}{x(1 + \sqrt{q}) - 2c} + \frac{1}{2} \log \dfrac{c}{c - x - kx^2}}{\dfrac{1}{2\sqrt{q}} \log \dfrac{\frac{1}{2}x(1 - \sqrt{q}) - 2c}{\frac{1}{2}x(1 + \sqrt{q}) - 2c} + \frac{1}{2} \log \dfrac{c}{c - \frac{1}{2}x - \frac{1}{2}kx^2}} \tag{6}$$

in which q stands for $(1 + 4kc)$.

From this latter expression we may attempt to find the proper values of k and κ in (1) by solving (1) and (6) simultaneously. To use the system of which the time-dilution curve was figured in fig. 2, from the position of the asymptote we have $(x + kx^2) = 0 \cdot 028$. Hence $k = (0 \cdot 028 - x)/x^2$. Substitute this value in (6) and the equation contains two values of t, a value of x and a value of c.

A suitable concentration for experimental work is now selected and a percentage hæmolysis curve obtained as in (1). The concentration c is known, and from the curve t_{100} and t_{50} can be at once obtained; this leaves only x as an unknown in (6) and the value can thus be found. In the example under consideration the experimental concentration $c = 0 \cdot 03$ was selected for the determination of the percentage hæmolysis curve: t_{100} was found to be 9 minutes and t_{50} $2 \cdot 15$ minutes; this gives $t_{100}/t_{50} = 4 \cdot 2$ approximately. Values of x have therefore to be inserted into (6), from which k is eliminated, so as to make the right-hand side equal to 4·2. The value $x = 0{\cdot}02$, giving $k = 20$, gives this figure, but unfortunately so does the value $x = 0{\cdot}028$, giving $k = 0$, and, indeed, almost any value of x between $x = 0{\cdot}028$ and $x = 0{\cdot}01$. This is merely another demonstration of the fact that (1) cannot be distinguished from (2) experimentally, for we now find that (5) cannot be distinguished from (6). Equation (5) is therefore sufficient for the treatment of the percentage hæmolysis curves of saponin and allied lysins, just as (2) is sufficient for their time-dilution curves.

The degree of skewness, as measured by the ratio t_{100}/t_{50}, given by (5) varies with the concentration of lysin employed; the extent of the variation is shown by the following figures, calculated for $x = 0{\cdot}028$. A few experimental values are also given for experiments in which the hæmolytic system showed an asymptote at this figure.

c.	t_{100}/t_{50} calculated.	t_{100}/t_{50} observed.
0·2	2·06	—
0·1	2·18	—
0·05	2·5	2·6
0·04	2·78	2·9
0·033	3·39	3·5
0·030	4·3	4·2

When $t_{100}/t_{50} = 2$, the percentage hæmolysis curve is symmetrical. This condition is found, both in theory and in experiment, when c is large, and may be erroneously construed as indicating a reaction of constant velocity proceeding through a population which is normally distributed. With small values of c the skewness of the percentage hæmolysis curve becomes marked, a condition which is met with in experiment, and which has been described by ourselves (1) and by Brooks (2).

In the case of the percentage hæmolysis curves for such lysins as the soaps and sodium taurocholate, in which, as the lysis proceeds, there is evidence of an acceleration of the reaction, we have to consider the distribution of the cell resistances as asymmetrically distributed, and the underlying reaction as one described by (2). The reasons for these assumptions have already been stated.

At the moment the reaction between cells and lysin commences, the resistances of the cells are normally distributed; the liberation of cell contents as the result of the lysis of the less resistant classes reduces, however, the resistance of the remaining classes, so that, as lysis proceeds, we have a continuous alteration in the resistance distribution. This makes a very difficult problem, but a sufficiently accurate, though approximate, result can be obtained as follows.

Select any class of cells of the population. Its resistance at the beginning of the reaction, before cell-contents have been liberated, may be called x, for x is the quantity of lysin which would be required to be transformed before the cells of this class would be hæmolysed if the underlying reaction proceeded uninterfered with by secondary reactions. The resistance of the cells of this class is, however, altered by the liberation of cell contents by classes previously hæmolysed, and we can take it that the reduction of resistance is proportional to the amount of previously liberated cell contents. The new resistance, x', of the class of cells of initial resistance x is therefore determined by (a) the initial resistance x, and (b) the amount of cell contents liberated by all the less resistant cells. This quantity may be taken, as before, to be approximately kx. Now $x = \mathrm{F}(x)$, and

$$\mathrm{F} = k\int x\,.\,dx = kx^2.$$

The constant may be omitted, as it does not influence the form of the new distribution. The values of x of the old distribution curve are now projected on the parabola $\mathrm{F} = x^2$. The origin of the old distribution curve is the same as that of the parabola; let the value of x giving the resistance of the most resistant cells be x_{100}.

The point on the parabola whose ordinate is equal to half the ordinate at x_{100} is now selected; its abscissa gives the position of the mean of the new distribution relative to the extremes, which are x_0 and x_{100}; the new values of x' are, of course, on a different scale to the old values of x.

This treatment shows that the median of the new distribution curve is situated, not midway between the extremes as in the initial distribution, but nearer to x_{100}, the upper extreme, its position being given by $0{\cdot}7175x_{100}$, instead of by $0{\cdot}5x_{100}$, as in the symmetrical curve. If a reaction described by (2) proceeds through such a distributed population we shall have

$$\frac{t_{100}}{t_{50}} = \frac{\log \dfrac{c}{c-x}}{\log \dfrac{c}{c-0{\cdot}7175x}} \qquad (7)$$

in which x is the amount of lysin used up in producing complete hæmolysis, and is the same as x_{100} above. The proper value in any given case is determined from the asymptote of the time-dilution curve for the lysin acting on the same suspension under identical conditions.

In the following table are shown the values of t_{100}/t_{50} calculated from (7) for various values of c; x has been taken as 0·35, a usual figure for sodium taurocholate acting on a suspension of standard strength at 25° C. Beside them are placed some experimental values which we have obtained from time to time. Observation of the course of the reaction when $c = 1 \cdot 0$ or more is, of course, impossible unless at very low temperatures.

c.	t_{100}/t_{50} calculated.	t_{100}/t_{50} observed.
2·0	1·46	—
1·0	1·52	—
0·5	1·75	1·8
0·4	2·13	2·0
0·38	2·37	2·4

When the reaction is brought about by high concentrations of lysin, we obtain percentage hæmolysis curves which present a positive skewness which cannot be greater than 1·4 in the measure which we employ. Lower concentrations of lysin give curves more nearly symmetrical, and there must be one concentration which gives $t_{100}/t_{50} = 2$, or symmetry. Lower concentrations still give percentage hæmolysis curves with a negative skewness, the curves appearing like those in which saponin is the lysin. These findings are in accordance with experimental experience.

Finally, we may refer to the curves described in (1). The percentage hæmolysis curve for saponin which is shown in that paper has $t_{100}/t_{50} = 2 \cdot 3$ approximately, and the initial concentration of lysin is 0·04. In view of the fact that the suspension used was a weak one, this value is quite to be expected, although it is perhaps rather small. The curve for sodium taurocholate gives $t_{100}/t_{50} = 1 \cdot 3$, with an initial concentration of 0·5 (printed, by an obvious misprint, as 1 in 7,000 instead of 1 in 4,000; the former dilution would not produce lysis, and the error is evident from the data on the next page). The value of x is 0·3, and the calculated value of t_{100}/t_{50} is accordingly 1·4. In connection with these curves two minor points should be noted. The latent period should be ignored and the measurement of t_{100} and t_{50} made from the origin $t = 0$, for the underlying reaction commences then, and it should also be borne in mind that the observed times for 50 and 100 per cent. hæmolysis are subject to small errors, and that these may be taken into account when necessary.

While we have confined our considerations to the symmetry of the distribution curves and of the percentage hæmolysis curves, as this is their most important property, we should note that it is possible to fit the experimental curves with calculated values. The method of doing this, and the results obtained therefrom, we propose to leave for future treatment.

3. *The R-notation.*

In (6) a method was described by which two hæmolytic systems, each containing the same lysin, can be compared with one another, the comparison being effected by means of a resistance constant R, together with a small constant k, which, to prevent confusion with that constant k referring to the effect of the cell contents on the lysin, we shall now call k_R. As put forward in (6), the method was an empirical one; it can, however, be expressed in terms of the equations described above.

As an example, take the comparison of two systems, one containing saponin and cells, the other containing saponin, cells, and the inhibitory substance, sucrose.

The time dilution curve for the saponin-cell system is approximately

$$\kappa t = \log \frac{c_2}{c_2 - x_2},$$

and the curve for the saponin-cell-sucrose system is approximately

$$\kappa t = \log \frac{c_1}{c_1 - x_1}.$$

The method is to eliminate t by taking equal values; thus we get

$$\log \frac{c_1}{c_1 - x_1} = \log \frac{c_2}{c_2 - x_2}.$$

Now the saponin-cell-sucrose system is R times as resistant as the saponin-cell system—that is, R times as much lysin is needed to produce the same result. So

$$\log \frac{c_1}{c_1 - \mathrm{R}x_2} = \log \frac{c_2}{c_2 - x_2},$$

from which it will be plain that $c_1 = \mathrm{R}c_2$, which is the result obtained in many examples in (6, 7, 10, 11).

If we compare two expressions of the form of (1) in a similar way, there are two possible results:—(*a*) Suppose that in the reaction described by the one curve we have R times as much lysin used up by the cells as in the reaction described by the second curve, and that the liberated cell contents react to the same extent with the lysin in the one reaction as in the other. Then $x_1 = \mathrm{R}x_2$ and the asymptotes are given by $(x_1 + kx_1^2/\mathrm{R})$, $(x_2 + kx_2^2)$, for, by its definition, if x is doubled k is halved, and so on. Then equality between the two right-hand sides of the compared expressions is given when $c_1 = \mathrm{R}c_2$, the proper values of k being inserted into the two sides of the equality equation. The relation between c_1 and c_2 is again a straight line passing through the origin if produced.

(*b*) Suppose that, when the cell component requires R times the amount of lysin to produce the change which results in lysis, the liberated cell contents also have their reacting power with the lysin increased R times. Then if the two right-hand sides of the equation of the form of (1), which describe the reactions which are compared, be written out in full, it will be seen that a relation between the asymptotes in which $(x_1 + kx_1^2) = \mathrm{R}\ (x_2 + kx_2^2)$ will not give equality when $c_1 = \mathrm{R}c_2$. To take a concrete example, if in two such expressions $x_1 = 0{\cdot}02$ and $x_2 = 0{\cdot}114$, then, when $k = 20$, we have the asymptote of the first curve giving twice the value of c as the asymptote of the second curve, from which the quantity of lysin used up by cells and liberated contents together in the first reaction is twice that used up by cells and liberated contents in the second reaction. Equality occurs between the equated right-hand members when $c_1 = 0{\cdot}2$, $c_2 = 0{\cdot}112$, instead of when $c_1 = 0{\cdot}2$, $c_2 = 0{\cdot}1$. Similarly for other values of c_1; the points which give the necessary relation between c_1 and c_2 lie to the right of the line $\mathrm{R} = 2$, except at the point in which this line terminates and which has for co-ordinates the values of the positions of the asymptotes. This will be found to be the case whether R is greater or less than unity.

Under these circumstances, the relation between c_1 and c_2 is described, not by a straight line, but by a curve approximating closely to this line. The result of an attempt to pass a line through the points of this curve will be the production of a small intercept on one or the other axis, the intercept being an indication that the analysis by means of a linear expression is approximate only. This is the result described in the papers referred to in this section; the relation between c_1 and c_2 was found, in many cases, to be given sufficiently accurately by $c_1 = \mathrm{R}\ (c_2 - k_\mathrm{R})$, the positive sign applying when $\mathrm{R} < 1$ and the negative sign when $\mathrm{R} > 1$. The constant k_R was treated as an empirical constant whose value is of little importance. From the foregoing, it seems probable that it is an expression of the fact that the real relation between c_1 and c_2 is not linear, but is given by a flat curve. In the attempt to pass a line through the points of this curve the constant appears, its value and the sign attached to it depending on the general disposition of the experimental points and the degree of approximation reached.

4. *The Variations in Surface.*

The point in which the equations dealt with in this paper differ principally from the well-known expressions of chemical kinetics is in their containing a term relating to the surface at which the reaction takes place. This term is necessary because the reaction cannot occur generally throughout the system, but only at the red-cell surfaces. This idea of discontinuity of reaction must now be developed further.

Imagine a hæmolytic system in which there are very few red cells—only hundreds instead of millions—and in which each cell is separated from its neighbour by a great distance. In the presence of a considerable amount of lysin, molecules of lysin remote from the cell surface will not react with the cell, whereas molecules near the cell will react. Call the portion of surrounding fluid near the cell which contains the reacting molecules the zone of action round the cell. Each cell will then have its own zone of action, provided that the cells are sufficiently far apart, and within this zone of action will proceed a reaction described by one of the expressions (1) or (2).

Suppose more cells be added to the suspension; each new cell may then come to lie either within one of these zones or outside them. If the volume of the zones be v and the total volume of the fluid be V, the chance of such a cell, surrounded by its zone, entering another zone will be v/V. If it does not enter, it will not interfere with the reaction round other cells, but will establish a zone of its own, in which reaction will proceed uninterfered with by external cells. If it enters a zone, the surface of formation will be increased within the zone and the amount of lysin used up increased also, for two cells have to be hæmolysed instead of one.

If, then, we have two suspensions, the first containing half the number of cells in the second, and if the equation for the time-concentration curve of the first be equation (2), that for the second will not be

$$\kappa t = \frac{1}{2\mathrm{S}} \log \frac{c}{c - 2x}$$

but

$$\kappa t = \frac{1}{\mathrm{S} + \psi \mathrm{S}} \log \frac{c}{c - \psi x}. \tag{8}$$

where ψ is the probability that the cells will occupy positions in the fluid inside the zones of action. The value of ψ is, moreover, easily obtained, for it is v/V.

The asymptote of (8) is reached when $c = \psi x$. If, therefore, we have a series of time-dilution curves for a lysin acting on suspensions of different strengths, we can obtain the value of ψ, of v/V, and thence the value of v, the volume of the zones of action, from a consideration of the position of the asymptotes.

In (6) a series of such curves are described in detail for saponin acting on suspensions of different strengths. We now attempt to obtain, from the figures there given, the value of v. The figures are as below :—

Suspension compared to standard	Asymptote, as value of c_∞.
4	0·0350
2	0·0285
1 (standard)	0·0255
½	0·0237
¼	0·0222

Suspensions less strong than ¼ the standard were found to hæmolyse in scarcely less time than the suspension of ¼ strength itself, and so we shall assume that, in this suspension, the zones of action were all separate from one another. The surface of the suspension would then be denoted by S, the surface of the suspension of ½ strength by ψ_1 S, that of the suspension of standard strength by ψ_2 S, and the surface of the stronger suspensions by ψ_3 S and ψ_4 S, respectively.

Now, as the strength of the suspension is doubled on successive occasions, we have v doubled on successive occasions, and we should have $\psi_1 \mathrm{S} = v/\mathrm{V}$, $\psi_2 \mathrm{S} = 2v/\mathrm{V}$, $\psi_3 \mathrm{S} = 4v/\mathrm{V}$, and $\psi_4 \mathrm{S} = 8v/\mathrm{V}$. V is constant throughout, and so we obtain the following calculated and experimental results for the value of v, the volume of the zones of action.

Suspension.	v, experimental.	v, calculated.
½	0·067	0·067
1	0·148	0·134
2	0·284	0·268
4	0·576	0·536

The correspondence between the calculated results and the experimental findings is excellent, and indicates the correctness of the assumptions, without which it would be very difficult, if not impossible, to explain the experimental results. In order to show the correspondence more completely, we shall calculate the curves described in (6) and compare the calculated curves with the experimental ones. The result of so doing is shown in the table below and in fig. 5;

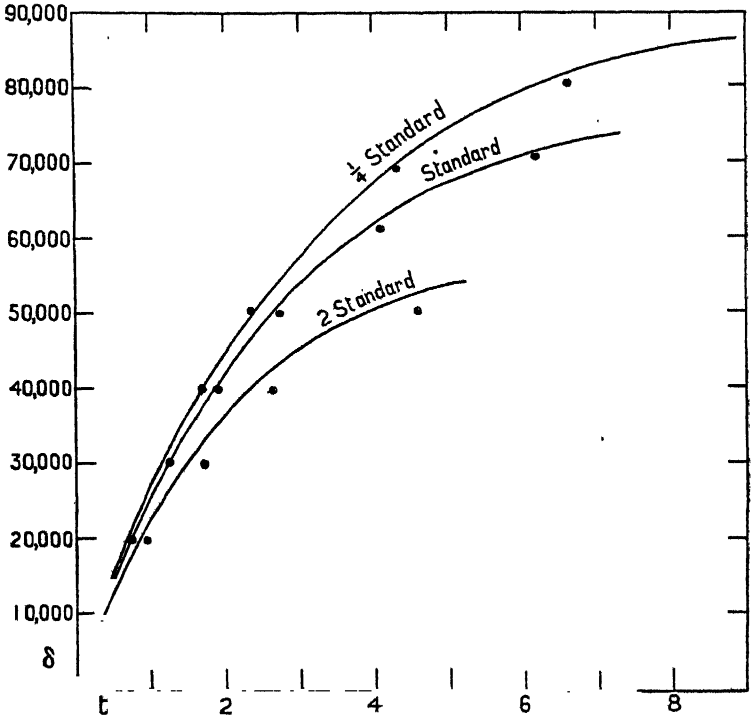

FIG. 5.

the value of v has been taken as $0{\cdot}07$ and the value of κ as $0{\cdot}33$. The value of x is determined in each equation by the position of the asymptote, which is obtained from the value of v/V.

$\frac{1}{4}$ suspension; $\psi = 0$, $x = 0{\cdot}0222$.

c.	t observed.	t calculated.
0·2	0·3	0·35
0·1	0·7	0·74
0·05	1·75	1·68
0·025	7·0	6·36

$\frac{1}{2}$ suspension; $\psi = 0{\cdot}07$, $x = 0{\cdot}0237$.

c.	*t* observed.	*t* calculated.
0·2	0·35	0·35
0·1	0·65	0·75
0·05	1·75	1·83
0·0$\dot{3}$	3·7	3·6
0·025	9·0	8·28

1 suspension; $\psi = 0{\cdot}14$, $x = 0{\cdot}0253$.

c.	*t* observed.	*t* calculated.
0·2	0·4	0·35
0·1	0·75	0·76
0·05	1·9	1·86
0·4	2·75	2·61
0·$\dot{3}$	4·0	3·75
0·27	6·0	7·29

2 suspension; $\psi = 0{\cdot}28$, $x = 0{\cdot}0285$.

c.	*t* observed.	*t* calculated.
0·2	0·42	0·36
0·1	0·85	0·79
0·05	2·4	1·99
0·04	3·3	2·94
0·03	7·5	7·02

4 suspension; $\psi = 0{\cdot}56$, $x = 0{\cdot}0350$.

c.	*t* observed.	*t* calculated.
0·2	0·45	0·37
0·1	0·9	0·83
0·05	2·6	2·31
0·04	4·2	3·9
0·037	7·0	5·3

The correspondence between theoretical and experimental results is very satisfactory, any marked differences being principally at parts of the curves where these are approaching the asymptotes and where the end-points are subject to a considerable error. We feel that our ability to calculate values so near to the values observed in this difficult experiment provides strong evidence for the correctness of the underlying assumptions.

Knowing the relation v/V, the size of the hypothetical zone of action can be calculated in absolute units. For example, in the suspension of standard strength, as usually employed in this series of papers, there are the cells from 1 c.c. of blood suspended in 20 c.c. of saline; of this $0 \cdot 4$ c.c. is added to $1 \cdot 6$ c.c. of lysin, to make a total volume in each tube of 2 c.c. Taking the number of cells in 1 c.cm. of blood as 5 million, there are 10^8 cells in each tube; each has a volume of $100 \mu^3$ approximately, and so the total volume of the cells in each tube is $10^{10} \mu^3$. The total volume of the fluid in the tube is nearly 2 c.c., or $2 \cdot (10^{12}) \mu^3$. For this suspension $v/V = 0 \cdot 14$ approximately, whence the radius of the zone of action round each cell is about $8 \cdot 8 \mu$. In the centre of this zone we imagine the cell to be situated, supposing it to be spherical in form, with a radius of about 3μ; the zone of action extends in all directions from the surface into the fluid for a distance of about 6μ. If the cell is taken as discoidal, this figure requires slight modification.

If the suspension is increased to between seven and eight times the strength of the experimental standard v/V becomes equal to 1; that is, it is certain that the cells will interfere with each other's zones of action, and the increase of the surface of formation S will be proportional to the number of cells in the suspension. This finding is in agreement with the fact recorded in (6), that with suspensions of this high degree of concentration hæmolysis is delayed much more than with less strong suspensions. Unfortunately, the end-points obtained with such strong suspensions are so bad that exact measurements cannot be made.

It is perhaps unnecessary to point out that the idea of the zone of action, although it explains the experimental results so well, may be nothing but a mathematical fiction, and that the real state of affairs may be somewhat different from that indicated. It is, for example, probable that this zone is not sharply defined, as we have taken it to be, nor of constant volume at all stages of the reaction. The value for its boundary is probably an average value for the point at which the slope of a concentration gradient becomes negligible and determined by several complex factors. The fact that the principles upon which the hypothesis is based are obvious ones, together with the accordance of the results with those of experiment and the reasonableness of the conclusions to which the conception leads, make us regard the present treatment as sufficient and satisfactory in its essentials.

It may be added that, although the hæmolysin saponin has been selected for the illustrative experiment, similar results are obtained, both in theory and in practice, with other lysins, such as the soaps, sodium hydrate and sodium taurocholate. In each of these cases there is similar evidence that the hypothetical zone of action exists and that the reaction on the cells does not occur continuously throughout the system.

5. *Conclusion.*

From the considerations dealt with in this communication it appears that an analysis of the kinetics of simple hæmolytic systems must take into account three principal factors: (*a*) the velocity of the reaction between the lysin and the cell component which is acted upon; (*b*) the distribution of the resistances of the cells in the suspension; and (*c*) the fact that the reaction does not occur continuously throughout the system but only in the region of the surfaces of the cells.

The reaction between the lysin and the cell component can be satisfactorily described by an expression similar to that applicable to chemical reactions of the first order, but containing also a term which relates to the surface upon which the lysin can act. Such an expression gives a remarkably close fit of calculated and experimental curves when the concentration of lysin is plotted against the time which it takes to produce complete hæmolysis or any given degree of hæmolysis.

The assumption that the resistances of the cells are distributed according to a symmetrical frequency curve, and that this distribution may be altered in some cases by secondary reactions, is enough to explain the form of the curves for percentage hæmolysis plotted against time—curves which are sigmoid in nature.

Allowance for the discontinuity of the reaction supplies the explanation, in a quantitative manner, of the curious relations which can be shown to exist between the activity of a hæmolysin and the strength of the cell suspension upon which it acts. This idea of discontinuity leads to the view that there is round each cell what we have called, with deliberate vagueness, a zone of action, within which molecules of the lysin enter into reaction with the cell surface. This zone extends for about 6μ from the cell surface in all directions, and its boundary is probably in actuality a point where the slope of a concentration gradient becomes negligible. A molecule of lysin situated outside this zone of action does not enter into the hæmolytic reaction proper.

In that we have come to the conclusion that the fundamental reaction between cells and lysin is one of the first order, we are in agreement with

Arrhenius and his pupils. At the same time our own previous criticisms of the findings of these writers is in no way altered, nor do we require to modify in any essential the criticisms of Brooks or of Fulmer and Buchanan. The writers who are thus criticised appear to have arrived at the correct result by unusual good fortune and by the process of cancelling one error with another. The first error lies in the failure to observe the sigmoid nature of the percentage hæmolysis curves, and the second the failure to take into account the frequency distribution. Indeed, it is not too much to say that, commencing with data from inaccurate experiment and applying a faulty analysis, they have arrived at a preconceived conclusion.

The results arrived at in this paper render the idea that the simple hæmolysins which are considered produce their effect by a solvent action of the red cell membrane, completely untenable, and show clearly that a chemical reaction is involved, accompanied by subsidiary reactions in which the contents of the cells, liberated by lysis of the less resistant classes, play an important part.

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The Heat Production of Nerve.

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Attempts have been made before (Rolleston (1) Hill (2)), but without success, to determine the heat produced by isolated nerve as the result of stimulation. Efforts also have been directed towards measuring the extra oxygen used (Haberlandt (3), Adam (4)), and the extra carbon dioxide eliminated (Tashiro (5), Parker (6), Moore (22)). The increase of oxygen consumption was too small to be determined with any certainty. The increase, however, of carbon dioxide elimination seemed to be measurable, though wide differences appeared between the results of Tashiro and of Parker. Prof. W. O. Fenn also has informed us that he has succeeded, by an independent method, in measuring the extra carbon dioxide set free by nerve during stimulation.

The following table summarises the relevant quantitative results available.

Table I.

Haberlandt.—*O_2 at rest.* Frog's sciatic, 19° to 24°; 0·0007 c.c. to 0·0014 c.c. per grm. per min.
Extra O_2, stimulated. Detectable, but no quantitative statement.

Tashiro.—*CO_2, at rest.* Spider crab, 0·0033 c.c. per grm. per min.; frog's sciatic, 0·0027 c.c. per grm. per min.
Extra CO_2, stimulated. Spider crab, 0·0046 c.c. per grm. per min.; frog's sciatic, 0·0043 c.c. per grm. per min.

Adam.—*O_2, at rest.* Frog's sciatic, 0·0008 c.c. to 0·0013 c.c. per grm. per min.
Extra O_2, stimulated. Frog's sciatic, never greater than 0·0013 c.c. per grm. per min.; usually much less.

Parker.—*CO_2, at rest.* Lateral line nerve of dogfish, average, 0·0046 c.c. per grm. per min.; frog's sciatic, average 0·0044 c.c. per grm. per min.
Extra CO_2, stimulated. Lateral line nerve of dogfish, average 0·00075 c.c. per grm. per min.; frog's sciatic, average 0·0006 c.c. per grm. per min.

Thunberg (26).—Frog's sciatic. *O_2 at rest.* (In air) 0·0004, (in O_2) 0·0006 c.c. per grm. per min.
CO_2 at rest. (In air) 0·0004, (in O_2) 0·0005 c.c. per grm. per min.

Thus, at rest, the following values have been obtained:—*Oxygen*, 0·0007 to 0·0014 (Haberlandt), 0·0008 to 0·0013 (Adam), and 0·0004, 0·0006 (Thunberg); *Carbon dioxide*, 0·0033, 0·0027 (Tashiro), 0·0046, 0·0044 (Parker), and 0·0004, 0·0005 (Thunberg). During stimulation the values are: *extra oxygen*, detectable (Haberlandt), not greater than 0·0013 (Adam). *Extra carbon*

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dioxide, 0·0046, 0·0043 (Tashiro), 0·00075, 0·0006 (Parker); a wide divergence between Tashiro and Parker, and approximate agreement (so far as Adam's results go*) between Parker† and Adam. It will be shown below that during prolonged stimulation the sciatic nerve of a frog gives out between 0·003 and 0·006 gram-calorie (of extra heat) per grm. per minute, an amount which, if obtained from the oxidation of glycogen, would necessitate the use of 0·0006 to 0·0012 c.c. of oxygen per minute, and the liberation of a similar amount of carbon dioxide.

The experiments (2) of A. V. Hill in 1912 were unsatisfactory in two respects: (*a*) no direct calibration of the thermo-electric apparatus was carried out, and (*b*) no attempt was made to measure the heat, if any, liberated *after*, as distinguished from *during*, stimulation.

With regard to (*a*), the method of calibrating by a small alternating current of known strength, passing through the tissue on the thermopile, was devised only at a later date (10). Experiments have shown that with a thermopile reasonably well insulated, and with a galvanometer reacting comparatively slowly (the inevitable consequence of the necessary sensitivity) there is a considerable difference, especially when the amount of tissue involved is small, between the actual value of one scale division of the galvanometer, in terms of heat liberated, and the value calculated from the number of junctions and their thermo-electric force. This is due (i) to the heat-capacity of the thermopile which may be comparatively large compared with that of the tissue; and (ii) to heat-loss to the surroundings or the cold junctions, which cannot be avoided, and occurs before the maximum deflection of the galvanometer is attained. It will be shown below that neglect of this calibration would introduce a four- to eight-fold error into the results recorded here.

With regard to (*b*), the existence of the recovery heat-production in muscle (7) was not realised till 1913, and it was not supposed in 1912 that an important part of the total heat due to activity might be liberated by oxidative processes occurring long after stimulation. In muscle the delayed heat due to oxidative recovery is about 1·5 times the initial heat (Hartree and A. V. Hill (8)), and its neglect would make the heat calculated from the initial deflection only 40 per cent. of the true value. In the case of nerve the study of this delayed heat is technically difficult, owing to the relative instability (when taken over long periods) of the "zero" of a system possessing the extreme sensitivity required for such experiments; only recently has it become possible. To our astonishment the delayed heat has amounted, not to 1·5 times the initial heat as in muscle, but to about ten times. Thus the delayed heat is the preponderant factor; this fact, together with the absence of a direct calibration, largely invalidates the conclusions drawn from the earlier experiments. A preliminary note on the present observations, published recently by one of us (9), also takes no account of the recovery heat, which was not measurable before the adoption of the thermal method of amplifying galvanometer deflections described below; the results, therefore, recorded in that note must be interpreted with this limitation in mind.

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* He stated that similar results could be obtained by "stimulating" wet twine.

† *Added in Proof.*—Prof. Parker informs us that it is probable that all his readings are, owing to the calibration of his apparatus, about 40 per cent. too high. If this be so—the matter is now being investigated—the appropriate correction must be made to all his figures.

Experimental Method.

Since 1912, the date of the last attempt to measure the heat production of stimulated nerve, many improvements have been made in myothermic technique, which is applicable as it stands to the present problem, but with the great advantages (i) that the stimulus can be localised far away from the thermopile, and made much smaller, with less danger of electrical leaks; and (ii) that the nerve does not move when excited, and so possibly cause errors due to change of position on the thermopile. The most notable improvements are as follows:—

(1) *Calibration.*—The introduction of a method of direct calibration, by which 1 mm. deflection of the galvanometer can be expressed directly in absolute heat units, without any doubtful calculations from (i) the number of junctions, (ii) their thermo-E.M.F's, (iii) the resistance of thermopile and galvanometer, and (iv) the sensitivity of the latter.

(2) *Galvanometer.*—The first step in this respect was the construction by one of us (A. C. D.) of a small and simple moving magnet galvanometer of the Thomson type, employing an extremely light, sensitive, and stable magnet system, and giving a "figure of merit" at least 10 times as great as that of any instrument previously available in this country (17). It was with this instrument that the first definite results were obtained, though the extreme sensitivity required of it made individual observations relatively uncertain. The stability of this galvanometer was then improved by the use of the alloy "Mumetal" for magnetic shielding, and an attempt was made to avoid mechanical vibration by floating it on a bath of mercury, in the manner described by Einthoven (11). Better results were now obtainable with it, but the high sensitivity required still entailed, amid the mechanical and

electrical disturbances of London, an instability of zero which prohibited observation of anything but the initial deflection.

(3) *Thermal Amplification.*—It was decided, therefore, to attempt to amplify the deflection of a stable moving coil galvanometer by a method suggested to one of us by Messrs. Kipp, of Delft, who were at the time engaged in the preliminary stages of manufacture of the thermo-relay described by Moll and Burger (12). A temporary arrangement of this kind was succeeded by a thermo-relay very kindly given to us by Messrs. Kipp. By this means it became possible, employing the mercury bath and suitable precautions, to work at a sensitivity of 1 mm. $= 2 \times 10^{-12}$ amps., on occasions with a relatively stable zero.

(4) *Thermopile Chamber.*—The complete immersion of the chamber containing the nerves, in well-stirred water in a double-walled vacuum flask, has greatly enhanced the temperature stability.

(5) *Thermopile.*—The use of the electro-plating method of W. Hamilton Wilson (13) in the construction of the thermopiles has made it possible to employ many more couples, and of far smaller heat-capacity, than was practicable with the older method of soldering the wires together.

We will consider these technical points more fully as they are the essence of the problem.

(1) *Calibration.*—In order to liberate a known amount of heat in a tissue lying on a thermopile, it is necessary to pass a known alternating current through it for a known time, and to measure its resistance. A difficulty is to determine the strength of the alternating current with sufficient accuracy; it is of the order only of 30 micro-amperes, and no ordinary method can measure such an alternating current at all. At first a measured direct current was converted by a commutator into an alternating one and passed through a potential divider, from which a suitable small alternating E.M.F. could be taken off. This method, simple enough in theory, produced in practice (at the sensitivity required) such disturbances that it had to be given up. The cause of the disturbances it was not possible to trace; often, in the course of these experiments, it has been necessary to discard certain methods otherwise satisfactory, on account of the disturbances associated with them, without any reasonable explanation of their cause.

A more satisfactory method was then tried, by which a small alternating current produced by an induction coil, or—better—by a valve-generator, was passed through the tissue and measured by means of a " vacuo-junction " of the most sensitive type, supplied by the Cambridge Instrument Company. The " vacuo-junction " consists of a very fine resistance wire inside an evacuated glass bulb, a thermo-junction being soldered to the wire. The thermo-E.M.F., which is proportional to the square of the current running in the wire, is then read by a galvanometer. No ordinary galvanometer is sensitive enough for this purpose; it is necessary to employ the very sensitive galvanometer, or thermo-relay combination, used for the experiments on the nerve. The vacuo-junction is calibrated with a small and accurately measured direct current (*e.g.* 2 volts through 70,000 ohms). The following formulæ may make the matter clearer :—

Let V = voltage of battery employed for calibrating hot wire.
R_1 = resistance in series with hot wire during direct-current calibration ; the wire is of negligible resistance in comparison with R_1.
x_d = deflection from vacuo-junction, for passage of direct current V/R_1 for some given time.
x_a = deflection from vacuo-junction, for passage of unknown alternating current for the same time.

Then C^2 for alternating current $= (V/R_1)^2 x_a/x_d$.

This method yields very accurate and consistent results.*

It was then necessary to find the resistance of the nerve between the electrodes. This was done by substituting for the nerve, in the alternating-current circuit, a resistance box reading to 100,000 ohms. A resistance which gave the same deflection on the vacuo-junction as when the nerve was in the circuit was taken to be the resistance of the nerve. This was rapidly ascertained by trial, and very consistent results were obtained.

Having determined the strength of the alternating current from the coil, or the valve-generator, when passing through the nerve, and the resistance R of the latter, it was now necessary to pass the alternating current through the nerve for a known time t_c seconds and to note the deflection D_c mm. We have :

$$D_c \text{ mm.} = R\,(V/R_1)^2\, x_a t_c/x_d. \quad \text{Joules.}$$
$$1 \text{ mm.} = R\,(V/R_1)^2\, x_a t_c/x_d\, D_c. \quad \text{Joules.}$$

From this, 1 mm. deflection on the scale can be expressed directly in absolute energy units.

The sensitivity of the galvanometer, or of the thermo-relay combination, may vary somewhat from time to time, and it is necessary to take account of this. For this purpose a potential divider was arranged to give any one of the following E.M.F.'s: 10^{-7} volt, $3{\cdot}33 \times 10^{-7}$ volt, 10^{-6} volt. On throwing this into the galvanometer-thermopile circuit (usually for 20 seconds) a deflection was obtained from which a sensitivity S_c, defined as millimetres deflection per microvolt, could be obtained. Introducing a factor $1/4{\cdot}18$ to turn Joules to calories, and the mass M of the nerve between the electrodes, we arrive at the following :—

$$1 \text{ calorie per grm. of nerve} = \frac{4{\cdot}18 \times M x_d R_1^2 D_c}{S_c V^2 x_a R t_c} \text{ microvolts.}$$

This number is very constant from experiment to experiment, having always a value of about 1,800 microvolts (with thermopile II) when employed with about 0·1 grm. of nerves.

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* In some of the earlier experiments a vacuo-junction was employed which was found to act as a potential divider, by reason of the two wires of the couple not being soldered exactly opposite one another on the resistance wire. This caused an error, the potential difference between the junctions adding to, or subtracting from, the thermo-E.M.F. This error was detected from the fact that a direct current gave different readings if passed in opposite directions. For larger currents the error would probably be negligible ; for the present purpose, however, it was too great to be permitted, and another vacuo-junction was substituted which did not show it.

In the experiments on the live nerve let D_l mm. be the deflection, with galvanometer sensitivity S_l, after stimulation for t secs. Then the heat production, calculated from the deflection D_l, is

$$\frac{S_c V^2 x_a R t_c D_l}{4 \cdot 18 \times S_l R_1^2 x_d D_c M t} \text{ calories per grm. of nerve per sec. of stimulation.}$$

The thermopile used may be shown, by experiments with an alternating current passing through the dead nerve, to "integrate" the heat effectively for several seconds, *i.e.*—with a constant alternating current—to give deflections proportional to its time of passage. Within limits, therefore, the method described above would be beyond reproach, were the heat of the live nerve to be liberated *only* during stimulation. In the case of muscle, the maximum deflection represents very accurately the initial heat production, except (*see* Hartree (14), *p.* 259) when that is very prolonged: the slow rise of the recovery heat production (8) enables a sharp distinction to be drawn between the "initial" and the "recovery" phases. This distinction is by no means so clear, if indeed it exists at all, in the case of nerve. As is shown by an approximate analysis given below, the heat production has no obvious break at the end of stimulation, but continues for several minutes. The heat, therefore, as calculated from the maximum deflection, contains an arbitrary factor, depending upon the speed of response of thermopile and galvanometer; the more rapidly the deflection occurs the less will be the heat liberated up to the time at which the maximum is reached.

There are two methods of circumventing this difficulty:—(A) To carry out a complete analysis of the heat production, employing a control curve as in muscle (*see* (14), (15)); this is very laborious, but necessary if the true time-course of the heat production is to be ascertained; (B) to work with the *area* of the deflection-time curve as described by Hartree and Hill (16). This latter method gives an accurate measure of the total heat, however distributed in time, and with comparatively little labour. It will be described rather more in detail.

In a system consisting of galvanometer, thermopile, and tissue, all the processes connected with the conduction of heat, the development of a thermo-E.M.F., and the deflection of the moving system, are governed by linear differential equations with constant coefficients. To solve these equations, even to determine the constants, would be an impossible task. This, however, is not necessary; the application of a "control" curve, obtained by "instantaneous" heating, to the "live" curve of deflection against time, enables a complete numerical analysis of the heat production to be made. This fact, well established now—apart from any theoretical considerations—by the consistency of many analyses performed, has an important corollary; the *area of a complete deflection-time curve is proportional to the total heat liberated, however distributed in time.* If, therefore, we know (*a*) the area of the deflection-time curve in a calibration experiment, in which a known amount of energy is liberated in the tissue as heat, together with (*b*) the area of a similar curve from the stimulated live nerve, then we may immediately calculate the total heat liberated by the latter over the period in question.

Let A_c be the area (in mm. × secs.) of the "calibration" curve, obtained by electrical heating for t_c seconds. Let A_l be the area of the "live" curve, obtained by stimulating for t seconds. Then with the same symbols as before the total heat is,

$$\frac{S_c V^2 x_a R t_c A_l}{4.18 \times S_l R_1^2 x_d M A_c} \text{ calories per grm.,}$$

or reckoned per sec. of stimulation,

$$\frac{S_c V^2 x_a R t_c A_l}{4{\cdot}18 \times S_l R_1{}^2 x_d M A_c t} \text{ calories per grm. per sec.}$$

It should be noted that the areas in question are total areas, for curves starting from the base line and returning to it again.*

The analysis of the heat production was carried out by the usual method, employing a control curve obtained by electrical heating during a comparatively short interval. Curves were recorded, either by plotting from readings made at known times, or photographically, chiefly the former.

(2) *Galvanometer.*—Owing to the unsatisfactory behaviour, as regards stability and "figure of merit," of the high-sensitivity galvanometers available commercially, we were forced to construct instruments for ourselves. In designing them, we received considerable help (*a*) from Prof. Paschen, then of Tübingen, now of Charlottenburg, who emphasised the great importance (for the avoidance of mechanical disturbances) of geometrical and mechanical accuracy in constructing the moving-magnet systems; (*b*) from Mr. Dye, of the National Physical Laboratory, who called our attention to the high retentivity of cobalt-steel and supplied us with a piece of it; (*c*) from Dr. C. V. Drysdale, who brought the alloy "mumetal," of very high initial permeability, to our notice, as a means of shielding from magnetic disturbances; and (*d*) from the Gutta Percha Company, who have given us every help in the construction of "mumetal" shields.

The figure of merit of the Paschen galvanometer previously available was only about 1500, and it showed serious magnetic and mechanical disturbances when sensitive. By various means: (*a*) reducing the size of the galvanometer and its coils and generally simplifying its design; (*b*) using very fine magnet systems of cobalt-steel; (*c*) mounting the magnets as accurately as possible on straight glass rods; (*d*) suspending these centrally by fine quartz fibres; and (*e*) employing small, well-silvered, flat mirrors about 0·05 mm. thick (supplied by Messrs. Halle Nachf., of Berlin-Steglitz); we have succeeded in pushing the figure of merit on occasions to 35,000, *i.e.*, in increasing the sensitivity more than 20-fold for a given deflection time. The instrument is described elsewhere by one of us (A.C.D. (17)). The thermopiles employed have been of moderately high resistance (170 and 700 ohms respectively), so the coils of the galvanometer were wound with a large number of turns of fine wire to have a total resistance of the order of 100 ohms.

Working in London in the neighbourhood of road traffic, electric motors and railways, and amid the usual activities of a large laboratory, we were troubled with a variety of disturbances. *Mechanical* disturbances were, to some degree, overcome by digging a hole through the floor of our cellar into the gravel 1½ metres beneath, and building up a large concrete pillar, weighing about 3 tons. A dish of mercury placed upon this showed continual rapid vibration, caused either by distant traffic or by movements in the laboratory. To avoid this we constructed an anti-vibration stand, consisting of a large sheet of heavy glass, loaded with iron masses to give it inertia, floating upon a bath of mercury, and held in place by pads of cotton-wool (see Einthoven (11)). This stand abolished almost completely the vibrations visible on a dish of mercury placed upon it; it was found, however, to have little effect upon the stability of this particular galvanometer. Possibly, rapid and irregular vibrations of small amplitude have no effect upon a well-constructed magnet system of relatively long natural period. The mercury stand, therefore, was temporarily discarded. *Magnetic disturbances* were far more troublesome. For some time we had used two large bell-shaped shields of dynamo-magnet steel. The permeability of such steel is high for large values of the field; for small values, however (such as all ordinary magnetic disturbances are), the permeability appears to be very low, and only about 90 per cent. of an external disturbing field was cut out even by the two shields together. This left large, irregular and uncontrollable movements of the galvanometer. The alloy "mumetal" made by the Gutta Percha Company, for "loading" their telegraph cables, is stated to have an "initial" permeability (*i.e.*, at very low fields) about 20 times as great as ordinary soft iron; by the kindness of the Company we were enabled to prepare a shield of mumetal tape, wound alternately with copper tape on a copper cylinder, which, weighing only about 2½ kilos., cut out about 99·9 per cent. of all external magnetic fields (See (24).) This shield, placed round the galvanometer, and then surrounded by a large bell-jar of dynamo-magnet steel, seemed to cut out nearly all magnetic disturbances. *Electrical disturbances,* due to leaks of current from the power mains and other similar causes, then proved troublesome. When one is dealing with a sensitivity of 1 mm. $= 5 \times 10^{-12}$ amp., even a million megohms insulation resistance will not eliminate the effect of a leak from 220 volt mains; in damp weather such disturbances became intolerable. The cellars, therefore, were re-wired inside properly-earthed steel tubes, while the walls and ceiling were covered with chicken-wire of 1-inch mesh, and the tables and galvanometer pillar with zinc sheet, all carefully earthed. By such means, and by carefully insulating the leads, the electrical disturbances occurring at extreme sensitivity were largely eliminated.

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* A set of typical values of the quantities used in the above equations is :—

Thermopile II, resistance 700 ohms.

$S_c = 350$, $V = 2{\cdot}0$, $X_a = 33$, $R = 40000$, $t_c = 2$, $A_l = 9400$, $S_l = 350$, $R_1 = 70000$
$x_d = 46$, $M = 0{\cdot}141$, $A_c = 1560$, $t = 10$, $m_1 = 0{\cdot}085$, $m_2 = 0{\cdot}056$.

Working anywhere but in a great city these precautions would probably have proved adequate. Except, however, on special occasions and for short intervals (*e.g.*, early on Sunday morning) they were still insufficient to permit the adequate analysis of the effect they had served to establish. During an interval, therefore, in the experiments, while the galvanometer was undergoing alteration, an independent method of attaining a high sensitivity was tested, viz., that of thermal amplification of the movement of a medium-sensitive but very stable moving-coil galvanometer. This proved so very satisfactory that it was adopted as standard in all subsequent experiments, and no return has been made for the present to the sensitive moving-magnet galvanometer. Our experience, however, with the latter has been recorded here (*a*) because it may be useful to others, (*b*) because it did enable the heat-production of nerve to be established, and (*c*) because the moving-magnet galvanometer will probably be used as a *secondary* galvanometer (see below) in future experiments, to secure quickness of response.

(3) *Thermal Amplification.*—If the edge of a concentrated beam of light reflected from a galvanometer mirror be allowed to fall upon the junctions of a linear radiation thermopile, as used for spectroscopic work, than a small deflection of the galvanometer will throw more, or less, light upon the junctions, and so cause a relatively large change in the E.M.F. produced by the thermopile. This change may be recorded by a second galvanometer, which will show, amplified 1,000 times or more if desired, the movements of the first one. Since the E.M.F. of the thermopile is proportional to the radiation falling upon it, which is inversely proportional, per unit of surface, to the *square* of the distance from the mirror, while the displacement of the light for a given rotation of the mirror is directly proportiona to the *first power* of the distance, it follows that a greater effect is produced in the secondary

galvanometer the closer the thermopile is to the primary one. In order to balance the E.M.F. produced in the thermopile by the light reflected from the primary galvanometer in its zero position, it is necessary to introduce an opposing E.M.F. into the circuit of the secondary. This may be done by exposing at a distance to the same source of light a second and similar thermopile, with a diaphragm in front of it, by which its constant E.M.F. may be adjusted: an important advantage of this method is that variations in the intensity of the light do not then cause fluctuations in the zero of the secondary galvanometer, since such variations produce equal and opposite effects in the two thermopiles.

The success of this method depends absolutely upon the use of a primary galvanometer of great stability, with good zero-keeping qualities: for all its perturbations (except rapid oscillations about a mean position, which average out) will be shown magnified on the secondary galvanometer. Preferably, also, it should possess a large mirror with good reflecting qualities, so as to provide as strong a beam as possible. Only a moving-coil galvanometer can possess these necessary characteristics, and we have employed an Ayrton and Mather galvanometer of the Cambridge Instrument Company, to which we have fixed, in place of its original heavy and rather poor mirror, a larger, lighter and better reflecting mirror supplied by Halle, of Berlin-Steglitz. The primary galvanometer has the following characteristics:—Resistance, 171 ohms; external resistance necessary for critical damping, 300 ohms approximately; period (to and fro) in the field, 7·6 secs.; sensitivity at 3 metres distance, 1 mm $= 4{\cdot}8 \times 10^{-10}$ amp. It will be seen how necessary amplification is, when it is realised that 20 seconds of stimulation of a live nerve, on the best thermopile hitherto constructed, would give, even at a distance of 3 metres, only about half a millimetre displacement. Actually, with the amplification usually employed, about 4×10^{-12} amp. in the primary would produce 1 mm. displacement in the secondary at the same distance, so that the effective amplification is about 120-fold.

To the eye, at any distance, the beam of light from this galvanometer, when standing on the pillar referred to above, appears to be perfectly still. Actually, when connected with the thermal amplifier, it shows random disturbances due to mechanical and electrical causes. The nature of the galvanometer prohibits a purely magnetic explanation of these. In order, as far as possible, to avoid mechanical oscillations, the galvanometer was placed upon the mercury stand (see above), while to eliminate electrical disturbances all the precautions outlined above (earthing the walls and tables and insulating the apparatus and leads) were adopted, together with such others as seemed from time to time appropriate.

Even so, disturbances do occur—chiefly, we believe, owing to two electric railways, each some 250 metres away. These disturbances, however, are far less, even at appreciably greater sensitivity, than in the case of the moving magnet galvanometer. They appear, moreover, to be purely random in character, and not due in any way to the experimental procedure.

Moll thermopiles were employed, supplied by Messrs. Kipp, of Delft, through the Cambridge Instrument Company. The one on which the beam of light fell was of the linear type, with a slit about 1 mm. wide in front of the junctions. This was placed about 75 cm. from the primary galvanometer, so that the actual displacement was magnified, not 120, but 480 times by the secondary. The edge of the beam of light was adjusted, in its zero position, to fall just half-way across the slit and immediately on the junctions; the E.M.F. produced by it in the thermopile was balanced, by means of an adjustable diaphragm, by the opposing E.M.F. of a Moll surface thermopile exposed at a distance to the same source of light (a 100-candle-power "Pointolite" lamp). In this position the sensitivity was at its maximum, and in consequence the scale most nearly uniform. At appreciable distances (one-sixth the width of the slit) on either side of this position the sensitivity became less and the scale ceased to be uniform.

The light was focussed by a large lens on to the mirror of the primary galvanometer, an adjustable slit being placed in the beam and focussed on the face of the linear thermopile.

In the primary galvanometer circuit, on the table in front of the observer, was placed an adjustable potential divider, whereby an E.M.F. up to 10^{-4} volt could be introduced to balance the E.M.F. of the nerve thermopile and to adjust the beam of light exactly on to the middle of the slit in its zero position. In actual use the adjustments are simple: the Pointolite lamp is turned on some time before readings are begun; the zero of the secondary galvanometer is noted; the nerve thermopile is thrown into the primary galvanometer, and the radiation thermopiles into the secondary; the secondary galvanometer is then brought back to its zero by adjusting the potential divider in the primary circuit, and the system is ready for use. To avoid disturbing the system, the galvanometers and relay were placed in a separate room, and the beam of light allowed to reach the observer through a hole in the door. Arrangements were made to facilitate adjustment without entering the galvanometer room.

No special properties are required by the secondary galvanometer; any low-resistance sensitive instrument should suffice. We have employed an Ayrton and Mather galvanometer made for us by the Cambridge Instrument Company and possessing the following characteristics: resistance, 31·3 ohms; external resistance for critical damping, 500 ohms; period (to and fro) in field, 6·3 seconds; sensitivity at 3 metres distance, 1 mm. = 4×10^{-10} amp. The only important objection to this galvanometer is its extreme slowness of movement when used with a low resistance in its external circuit—as is necessary for sensitivity. We have employed an external resistance of about 100 ohms in addition to the thermopiles; this sacrifices more than half the possible sensitivity, but enables rather quicker readings to be obtained. In respect of quickness of response it would be better to replace this galvanometer by the moving-magnet instrument described above, which at the same sensitivity would give a full deflection in less than a second. This we intend to do in later experiments.

Towards the end of the present series of experiments we received, from Messrs. Kipp, of Delft, a thermo-relay designed by Moll and Burger (12) with which we have replaced the rather complicated arrangement of thermopiles described above. This beautiful instrument consists of a single couple of constantan-manganin-constantan, about half a millimetre wide and 0·001 mm. thick, blacked with colloidal carbon, and mounted horizontally in a vacuum. If a vertical band of light coming through a narrow slit in front of the lamp be reflected from the mirror of the primary galvanometer, and allowed to fall exactly symmetrically on the central manganin strip, the junctions will be equally heated and no E.M.F. will be generated in the couple; if a slight deflection occur one junction will be heated more than the other and an E.M.F. will be set up. The sensitivity is stated to be independent of the sharpness of the band of light, and we can entirely confirm the claim that the E.M.F. is proportional to the displacement of the band. The sensitivity may be raised by bringing the relay closer to the primary galvanometer, and by concentrating the band by a cylindrical lens; also to some degree by adjusting the width of the band. The sensitivity is so great with the single couple employed (*a*) because there is no loss of energy from the couple through air around it, and the loss by conduction along the very thin strip employed is small; and (*b*) because the reflected light may be concentrated, by a cylindrical lens, on to the element. A quick secondary galvanometer is stated to follow the deflection of the primary one with less than 3 seconds' lag.

With this thermo-relay we have found it easy to obtain a sensitivity of about 1 mm. = 2×10^{-12} amp. A much higher sensitivity could obviously be reached if desired, either by employing a more sensitive secondary galvanometer, or even by introducing a second relay. Nothing, however, would be gained by so doing, since errors and disturbances would be magnified in the same proportion as readings. Perhaps in a quiet laboratory in the country the use of a higher sensitivity might be justifiable.

The readings of the arrangement as we have used it are necessarily slow, owing mainly to the slowness of the secondary galvanometer. For the fuller analysis of the time-course of the heat-production, which we propose to undertake later, it will be desirable to replace the secondary galvanometer by a quicker one, as suggested above. It may be possible also to use a primary galvanometer of shorter period. This will depend upon the stability and freedom from disturbances of such an instrument. For quickness of reading, as distinguished from convenience, sensitivity and uniformity, it may be advisable to return to the combination of thermopiles, in place of the vacuum thermo-relay, since the linear thermopile, being in air, more rapidly attains its full E.M.F. on exposure to light, its lower sensitivity per couple being partly compensated by the large number of couples.

(4) *Thermopile Chamber.*—Before 1919 myothermic experiments were conducted by means of separate thermopiles erected in a suitable moist chamber in the room. Since that date a great improvement in reliability and stability has been achieved by building the thermopile into a chamber of dimensions sufficiently small to allow of its being sunk under well-stirred water in a double-walled silvered vacuum flask. For most purposes the best type of chamber is that recommended by Fenn (18), which consists simply of a large glass test-tube, 4 or 5 cm. in diameter, closed at the top by a rubber bung which carries the glass rods holding the thermopile and accessories inside the tube.

It was supposed that the use of a silvered vacuum flask in place of the test-tube would still further shield the thermopile from temperature variations and ensure a greater stability of zero. The results were entirely disappointing. Equalisation of temperature between inside and outside became so slow, since it could occur only through the bung, glass rods and copper leads, that observation was not possible for very many hours after the chamber had been closed and placed under water; a steady creep of the zero persisted for long periods. Even with the glass test-tube this creep was bad enough; an interval of four or five hours was generally necessary before stimulation could be commenced; eight hours or more was better. In view of the possible deterioration of the nerves it was inadvisable to wait longer than was essential, and the use of the vacuum flask cover for the thermopile was discarded.

In order to secure greater stability of zero, once temperature equalisation is complete, it would probably be better to maintain the temperature of the water in the large vacuum flask constant by means of a regulator.

(5) *Thermopile.*—The introduction by Hamilton Wilson (13) of the method of constructing thermo-elements by electroplating, instead of soldering, has made possible a considerable increase in the number of elements which can be compressed into a given space, and a large diminution in the heat capacity of these. Moreover, the regularity and flatness with which a continuous wire can be wound and plated, make it unnecessary to use so thick a layer of insulating varnish, and avoid electrical leakage through possible small projections. There would seem to be no possibility of improving on the constantan-silver couples advocated by Wilson, and all the thermopiles constructed for the present investigation have employed constantan wire plated with silver.

It was obvious that the number of couples to which a set of nerves was exposed could be increased very largely by winding them on a number of wedges, the junctions lying along the sharp edges, and being arranged in series as in fig. 1. It is possible, by using No. 48 S.W.G. constantan wire (0·041 mm.), to wind, and electroplate, as many as 10 turns to the millimetre, without risk of short-circuiting. This, in a thermopile 12 mm. long would give 120 couples, with junctions at the thin and the thick edges of the wedge. By placing 12 such wedges in series, as in fig. 1, with their thin edges arranged round a semi-circular groove, it would be possible to expose the nerve to 1440 couples, which should provide a sufficient thermo-E.M.F. per 1° C. to allow any reasonable amount of heat to be detected with a sensitive galvanometer. The first attempt to construct such a pile was made by Prof. W. O. Fenn, working with one of us (A.V.H.), but with little success. A second attempt was made later by another of us (A.C.D.), but the instrument although constructed, as in fig. 1, showed so extremely low a sensitivity that there was obviously some fundamental defect in its design.

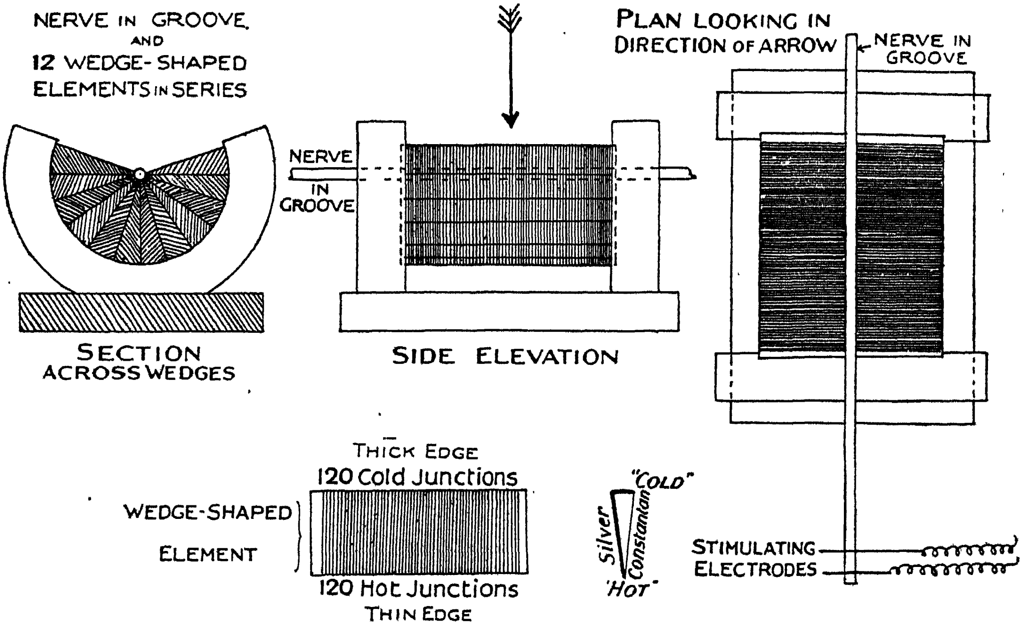

Fig. 1.—Thermopile with 12 wedges each carrying about 120 couples of constantan-silver. Hot junctions in a line along the edge of the wedge. Wedges arranged with hot junctions along surface of groove in contact with nerve.

The only defect we could see was the small length of each couple actually exposed to external heat, and we imagined that the junctions were never really warmed to the temperature of the tissue lying on the thermopile, owing to the rapid conduction of heat along the wires away from the point-contact at the junction. Another attempt, therefore, was made as in fig. 2. In this also wedges were employed, but their thin edges were filed away so as to present a plane surface about 1 mm. across. Owing to the width of this thin edge it was not possible to employ more than 3 or 4 wedges. The wire was wound on the wedge, and plated as shown in fig. 2, the junctions being arranged (i) at the middle of the thin edge, and (ii) at one of the back corners. The objects of placing the cold junction unsymmetrically were (*a*) to diminish the electrical resistance, silver having so much higher an

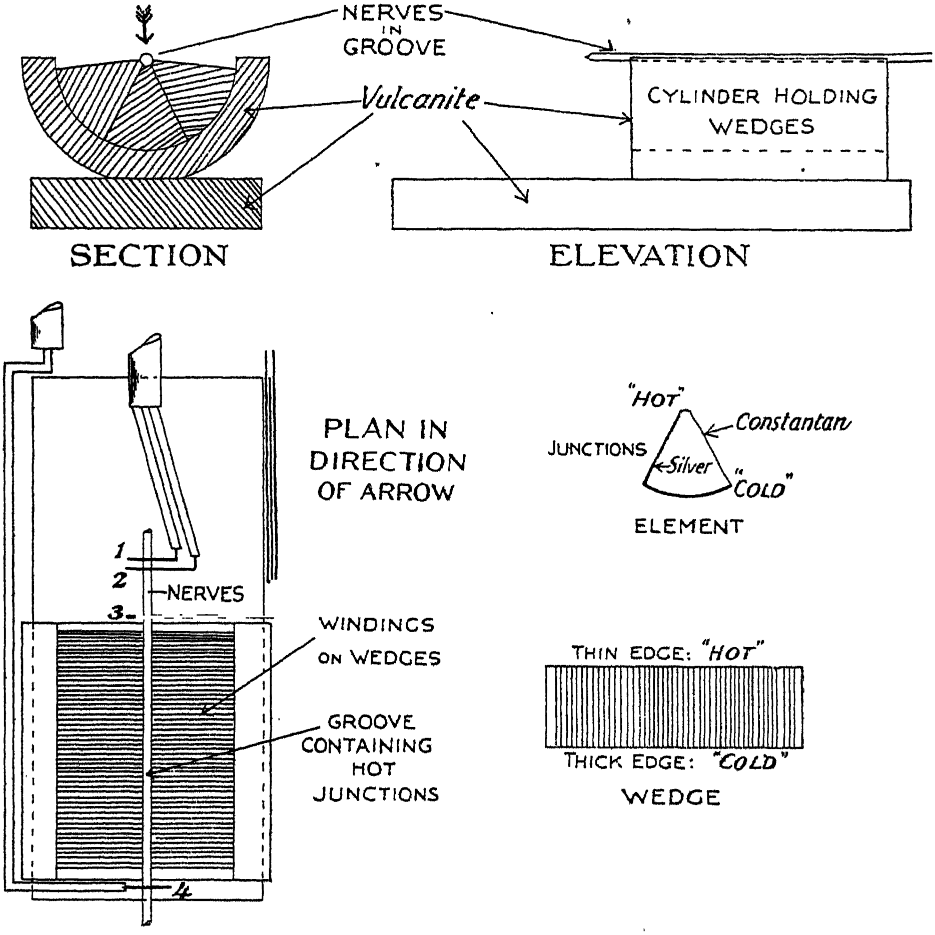

Fig. 2.—Thermopile with 3 wedges each carrying about 100 couples of constantan-silver. Thin ends of wedges filed away to allow exposure of hot junctions to nerve for an appreciable distance on either side. 1 and 2 : stimulating electrodes ; 3 : electrode in contact with live nerve ; 4 : electrode in contact with injured nerve. *Note.*—The position of electrodes 1 and 2 is as adopted in the original instrument. These were found to be much too close to the hot junctions of the thermopile and were removed to a greater distance, as shown in fig. 3. Calibration is carried out through electrodes 1, or 2, or 3, and 4. The action current is observed between 3 and 4. In the earlier experiments 3 was placed over the middle of the thermopile.

electrical conductivity than constantan, and (*b*) to diminish the heat conduction from hot to cold junctions, by increasing the length of the better conductor at the expense of the poorer. With this form of wedge each junction is exposed, for about half a millimetre on either side, to the nerves lying upon it, and trial immediately showed the very high sensitivity of the arrangement.

In the first thermopile (I) of this type there are three lines of junctions each about 12 mm. long, with about 7 couples per mm. of No. 46 S.W.G. constantan wire (0·061 mm.), a total of about 250 couples of resistance 170 ohms. The hot junctions in the groove were very carefully examined under a stereoscopic microscope and arranged with the utmost regularity and as flat as possible, then insulated with very thin layers of shellac and black varnish alternately, each being carefully dried before the other was applied, and the whole finished with two or three layers of dilute shellac. Before use, on every occasion, the surface was "waterproofed" by rubbing it with a brush previously wetted with a solution of paraffin wax in ether and then allowed nearly to dry. The polished smear of paraffin wax so obtained protected the shellac from the damp of the tissue, and probably saved the nerve from the slightly harmful effect (often observed with muscles) of the varnish. *The process of insulation has an importance which cannot be overstressed,* since we are dealing with such very small currents that a leak of any kind may largely affect the results. The layer of insulator must not be thick: as we have used it, it only slightly affects the rate at which the junctions pick up the temperature of the nerve upon them. If the insulation be in good condition the resistance between nerve and thermopile will be many megohms; if not, it should be cleaned off and renewed.

The second thermopile (II) has 4 wedges, each containing about 100 couples of No. 48 S.W.G. wire (0·041 mm.) in a space of about 12 mm., a total of 400 couples with a resistance of 700 ohms. This is little more effective than thermopile (I), and it is suspected that putting four wedges instead of three, and a larger number of couples per mm., has diminished the effective heating of the junctions; while the extra resistance has compensated for the extra number of junctions made possible by the finer wire. In a third thermopile (III) now completed and tested, but not used in the experiments recorded here, there are three wedges, 270 couples of No. 46 S.W.G. (0·061 mm.) wire, and a resistance of 270 ohms.

The silvering of the junctions is carried out very slowly, occupying an hour or more, and the total amount of silver deposited is regulated by the amount of current passed, according to the formula:—

> Number of ampere-seconds $= 25\ nlmd^2 \times 10^{-7}$, where n is the number of couples, l is the length to be silvered (in centimetres) in each couple, d is the diameter of the wire in 0·001 mm., and m is the number of times the conductivity of the silver sheath is to exceed that of the constantan core. We have usually taken the factor m to be 20.

One means to avoid disturbances is to remove from any possible electrical contact with the thermopile all wires or electrodes. The stimulating wires, therefore, are carried in a separate glass tube, and the platinum electrodes are free in the air. Four electrodes are employed, (1) and (2) for stimulating, (3) and (4) for picking up the action current when necessary, (1), (2) or (3) and (4) for electrical heating (calibration).

In all the thermopiles the wedges containing the elements were made of ivory, soaked in paraffin oil to waterproof it. In thermopiles (I) and (III) the rest was of vulcanite. In (II) an attempt was made to quicken up temperature equalisation, and to ensure greater temperature stability, by the use of brass for the semi-cylinder (figs. 1 and 2) carrying the wedges. This appeared to have no effect and in (III) we returned to the use of vulcanite.

When dealing with the very small amounts of heat liberated by a nerve on stimulation, precautions must be taken to avoid any error due to heat liberated by the stimulus itself.

To show the importance of this factor, if the faradic current from a Harvard induction coil (2 volts in primary), with the secondary removed as far as possible from the primary and then placed at right angles to it, be passed for a few seconds through the length of a dead nerve on the thermopile, easily measurable deflections will be obtained. If the stimulus be made just over-maximal, as is desirable during an actual experiment (*e.g.*, by removing the secondary as far as possible and tilting it up through 30°) and passed through the length of the nerve, the deflection produced by the stimulus itself in 2 secs. will be as great as that by the nerve in 20 secs. It is clearly impossible, on the scale of heat with which we have to deal, to employ a stimulus passing the whole length of the nerve, as is usually done in muscle. It is essential that the stimulus should be removed as far as possible from the thermopile, so that the heat produced by it shall be unable to reach the junctions. For this purpose the nerves employed should be long and the stimulating electrodes removed from the thermopile as far as the nerves will allow. The importance of this factor was not fully realised till after thermopile (II) had been constructed: (I) and (II) both have electrodes whose distance from the thermopile can be adjusted only by bending them about. In thermopile (III), (fig. 3), however, the electrodes are carried by a vulcanite tube which passes through a glass sheath into the chamber, and may be moved at will and clamped in any desired position by a vulcanite screw.

It is easy to test whether an appreciable error is introduced, with the electrodes in any position, by the heat liberated by the stimulus itself. All that is necessary is to kill the nerve with chloroform vapour, or other means, and to repeat the observations. If the electrodes be too close, or the stimulus too strong, appreciable deflections may be caused in this way. These have not the same character as those due to the heat-production of the live nerve since they occur only with a considerable delay, 60 to 120 secs., and with a slow rise and fall, owing to the time occupied in conduction of heat from electrodes to thermopile. They may, however, cause very serious errors, since (as we shall see later) nearly all the heat liberated by the live nerve as the result of stimulation is also considerably delayed. By allowing a sufficient gap between electrodes and thermopile this error may be made small, but to abolish it entirely we have introduced between the electrodes and the thermopile two comparatively large plates of silver, on which the nerves rest. Silver is harmless to the nerves, and its very high conductivity allows it to carry away and dissipate any heat reaching it from the electrodes, thus effectively "shielding" the thermopile. In thermopile (III) these silver plates are soldered below to brass blocks, in order to provide a greater heat capacity to the silver, and so still better shield the junctions.

The amount of nerve lying in the groove (20 mm. in thermopile (II)) is quite small, usually less than 50 mgrs. A rather greater sensitivity could obviously be obtained by employing more nerve, but even this amount requires the use of 6 or more English *temporaria* or 4 large Dutch *esculenta* frogs, (12 or 8 sciatic nerves respectively), and the careful preparation even of these takes a considerable time. In thermopile (III) the groove is rather larger, more nerves have to be employed, and a rather greater sensitivity is actually obtained.

After electrical heating of the dead nerve the galvanometer returns practically to zero (say to 4 per cent. of its maximum displacement) in about 1 minute; so that the thermopile must lose about 40 per cent. of the heat present at any time in the following 10 secs. After an "instantaneous" heating the galvanometer attains its maximum in about 8·5 secs. In that time nearly 40 per cent. of the heat will have been lost, which, together with the relatively large heat-capacity of the parts of the thermopile which have to be warmed by the nerve, serves to bring the sensitivity of the thermopile far below its theoretical value (*i.e.*, calculated from the number of junctions and their thermo-E.M.Fs.). To take an example,

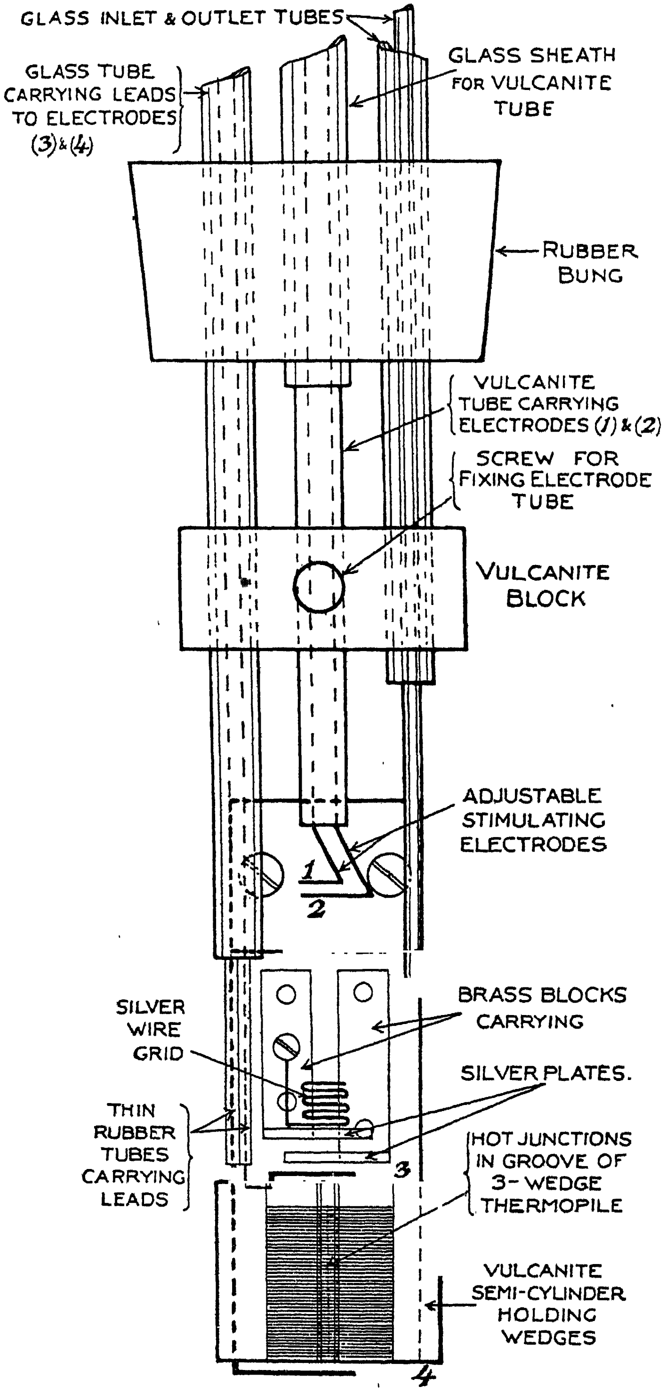
GLASS INLET & OUTLET TUBES
GLASS TUBE CARRYING LEADS TO ELECTRODES (3) & (4)
GLASS SHEATH FOR VULCANITE TUBE
RUBBER BUNG
VULCANITE TUBE CARRYING ELECTRODES (1) & (2)
SCREW FOR FIXING ELECTRODE TUBE
VULCANITE BLOCK
ADJUSTABLE STIMULATING ELECTRODES
1
2
SILVER WIRE GRID
BRASS BLOCKS CARRYING
SILVER PLATES.
THIN RUBBER TUBES CARRYING LEADS
HOT JUNCTIONS IN GROOVE OF 3-WEDGE THERMOPILE
3
VULCANITE SEMI-CYLINDER HOLDING WEDGES
4

Fig. 3.—Thermopile III, containing 3 wedges, together with silver plates and silver-wire grid to isolate thermopile from heat produced by the stimulating current (or any other cause operating in the upper half of the nerve). Electrodes 1 and 2 (for stimulation) adjustable to fit the length of the nerve employed. See also description of fig. 2. Natural size.

thermopile (II) with 400 couples of constantan-silver should have a thermo-E.M.F. of about 14,000 micro-volts per 1° C. Actually, the maximum deflection obtained from a nerve warmed electrically with a known amount of heat corresponds to about 1,800 micro-volts per calorie per grm. of nerve, $\frac{1}{8}$ of the full calculated value. In thermopile (III), with fewer windings, the result is better, viz., about $\frac{1}{4}$ of the calculated value.

Means are provided for the introduction of gas, or fluid, into the chamber when required. Most of the experiments have been performed in an atmosphere of oxygen.

In order to avoid electric leaks the thermopile should be kept as dry as possible.

Other experimental details. (*a*) *Stimulating Coil.*—Two induction coils of the Harvard Apparatus Company were employed. One of these had been equipped with a stronger spring to give more frequent stimuli: it provided about 140 make and 140 break shocks per second. The other was of the ordinary type, giving 50 make and 50 break shocks per second.

(*b*) *Valve Generator.*—This was supplied by the Cambridge Instrument Company, and is capable of giving a pure sine-wave alternating current, of frequency from 100 to 5,000 per second as required. This instrument will be employed later for studying the effects of frequency of stimulation. In the present series it has been used only for heating the nerve in calibration experiments; for that purpose a frequency of about 500 cycles per second was employed.

(*c*) *Resistance of Nerve.*—A few measurements were made of the resistance between the electrodes at opposite ends, as employed during calibration, but determined by the bridge method; the valve generator at moderate frequency as source of current was used, together with a high resistance telephone. The results agreed closely with those of the method described above. The observed resistance is rather greater with lower frequencies than with higher, owing probably to polarisation at the electrodes, but the error so introduced is not sufficient to influence the results. The specific resistance of nerves, calculated from its observed resistance R, its mass m, its length l, and its assumed specific gravity s (1·06), according to the formula $\mathrm{R}m/sl^2$ (Hartree and Hill (19)), has an average value in our experiments, at about 15° C., of 235, which is not far from that (175) found by Hartree and Hill for dead frog's muscle during similar calibration experiments.

(*d*) *Non-uniformity of Nerve Section.*—It was necessary, as described above, to employ long nerves, in order to remove the stimulating electrodes as far as possible from the thermopile. The sciatic nerve tapers considerably during its course, giving off many branches, and only the distal half (with no branches) lay upon the thermopile, the stimulus being applied to the extreme upper end. This causes no error in the "live" experiments, in which presumably the whole nerve enters into maximal activity and the rise of temperature is uniform throughout. In calibration experiments, however, using electrodes (1) or (2) and (4), the upper half being thicker has a lower resistance (r_1) than the distal half (r_2), so that the heat-production in the half lying on the thermopile is greater than calculated. It is obvious that the same deflection would be produced by the same current running through a uniform nerve of total resistance $2r_2$ and heating it uniformly throughout. Thus, for R in the formulæ given on p. 227 above we should really substitute $2r_2$ for $(r_1 + r_2)$. This will increase all the results calculated in the usual way in the ratio $2r_2/(r_1 + r_2)$. Since the resistance of a given length of nerve is inversely proportional to its mass, it is easy to determine the above ratio by weighing the two halves of the nerve separately: let these weights be m_1 and m_2; then the results calculated in the usual way must all be multiplied by $2m_1/(m_1 + m_2)$ to allow for the non-uniformity of the nerve.

In later experiments we placed electrode (3) just above the thermopile, and used (3) and (4) for calibration, thus ensuring a uniform stretch of nerve. The results agreed with those calculated, as above, using electrodes (1) and (4).

(*e*) *Action Current.*—For determining whether the nerve is alive, or for estimating its condition, a quantitative measurement of the action current on stimulation is useful. For this purpose the two electrodes (3) and (4), (3) on an uninjured point and (4) on the cut end, are employed, the monophasic current from these being led off, through a 10 mfd. condenser, to a high-resistance, sensitive, moving-coil galvanometer. The use of a condenser in the circuit avoids the necessity (i) of employing non-polarisable electrodes, and (ii) of balancing the injury current. Large deflections, for a few seconds of stimulation, are obtained in this way.

(*f*) *Nerves Employed.*—So far only the sciatic nerves of frogs have been used, in the earlier experiments those of winter English *temporaria* in rather poor condition, in the later those of spring Dutch *esculenta* in good condition. In all experiments four or more frogs, as large as possible, were pithed and the sciatic nerves dissected out from their exit from the spine to the bifurcation at the knee, branches being cut away a short distance from the trunk, and a thread tied at each end of the trunk. These were kept in Ringer's solution 30 to 60 minutes during the dissection and were then blotted for a moment on filter paper and arranged on the freshly paraffined dry thermopile. The central ends of the nerve were placed over electrode (1), the trunk being carried under electrode (2), then over the silver plates into the groove of the thermopile, under electrode (3), and with the ligatured distal end resting in the elbow of electrode (4). In the thermopile groove care was taken to cover the junctions along the side. The thermopile was then tightly fitted into a large test-tube wet with Ringer's solution. Moist oxygen was bubbled through, and the whole was immersed in well-stirred water in a Dewar flask.

Usually the dissection was performed the first thing in the morning and the observations begun about five hours later. In a few experiments the nerves were left overnight in position and observations begun next morning. In all the experiments recorded here the temperature was uniformly about 15° C. Observations were continued usually over a number of hours, during which time the heat responses gradually fell.

In many experiments the calibration procedure referred to above was carried out on the live nerve; the currents used for this are not excessive (no larger than for stimulation), so no harm is caused to the nerve. The time required for warming (1 or 2 seconds) is so short that the error introduced by the heat response of the live nerve is negligible compared with the heat due to the current itself. Calibration of the live nerve in this way was often controlled by calibration of the dead nerve afterwards, and no significant differences were found.

Results.

(1) *Absolute Values.*—More than twelve reliable experiments were performed, each involving a large number of observations often extending over more than a day. Details of the individual experiments will not be given. The results are consistent among themselves, as is seen from the range of values quoted.

Calculated simply from the maximum deflection (D_1) *the heat-production per grm. per sec. of stimulation ranged from* $5 \cdot 5$ *to* 10×10^{-6} *cal., with a weighted average of* $7 \cdot 6 \times 10^{-6}$ *cal.*

Calculated from the total area of the deflection-time curve the values ranged *from* $4{\cdot}3$ *to* $9{\cdot}7 \times 10^{-5}$ *cal. per grm. of nerve per sec. of stimulation, with a weighted average of* $6{\cdot}9 \times 10^{-5}$ *cal.*

The variation from one experiment to another is but slightly due to technical errors, as is seen from the fact that the number of microvolts given in the calibration per calorie per gram of nerve, involving many operations of the same kind as in the case of the nerve response, is constant in various experiments to within 10 per cent. On the other hand, in the live nerve, the ratio of deflection per second of stimulation to galvanometer sensitivity varies by almost 100 per cent., the lowest values as a rule being given by nerves that have been on the thermopile for a long time. Because of a possible deterioration with time before observations can be commenced, the values given above may be a trifle low, but apart from this it is difficult to believe that they are in error by more than 10 or 20 per cent.

Before going further, it is essential to consider the heat produced by the stimulating current itself. When passed through the entire length of the nerve for 1 sec. the current from coil (I), removed to its greatest distance and tilted to 30°, gives about as much initial *deflection* as the stimulated live nerve does in 10 secs.; while the total heats reckoned from the *area* are almost the same per unit of time, whether of stimulation or of passing the current. When the same coil position is used in stimulating at the far end of the nerve the resistance is only 1/7 as much, and the heating effect therefore is seven times greater (E^2/R). In a just-maximal stimulus, therefore, the heat liberated between electrodes (1) and (2) may be about seven times as great as the amount produced by the response of the nerve itself. That this heat, however, does not contribute to the quantity measured above, provided that adequate precautions be taken, is shown by the following considerations :—

(*a*) The heat recorded by the thermopile on stimulating the end of a live nerve is never reproduced after the nerve has been killed. With thermopile (II), which allows the stimulating electrodes to be placed more than 2 cms. away from the thermopile, a maximal stimulus, as employed during the live experiments, may be passed from the coil for 4 minutes or longer through the end of a dead nerve with no sign whatever of heat being conducted down. If the stimulus be limited to 20 seconds, the coil may be turned horizontal and moved several centimetres closer to the primary before any effect whatever is observed.

(*b*) In some of the earlier experiments, before precautions were taken, stimulus heat did appear in the live and the dead nerve alike. It was easy, however, to recognize it then, as it arrived 60 or more seconds after the stimulus and rose and fell gradually, giving a well-defined hump on the live heat curve, or a similar absolute deflection with the dead nerve.

(*c*) In one experiment all was prepared as usual, but a thread tied about the nerve at the top of the thermopile. Neither heat nor action current was obtained below the thread in spite of strong super-maximal stimuli. It was conceivable that in the live nerve there is considerable spread of the stimulating current, either directly or in the form of electrotonic currents within the nerve, which may be absent from the dead tissue as a result of decreased polarization, structural breakdown, and the like. This might have the effect of bringing the current heat closer to the thermopile in living than in dead nerve and so possibly producing an error. The above experiments show that this possibility may be discarded.

(*d*) The resistance, transverse and longitudinal, of the nerve between electrodes (1) and (2) was determined several times, and found to be the same before and after death.

(*e*) The energy in a stimulus is easily measured on the vacuo-junction, and as the coil is rotated from 90° to horizontal is found to vary approximately

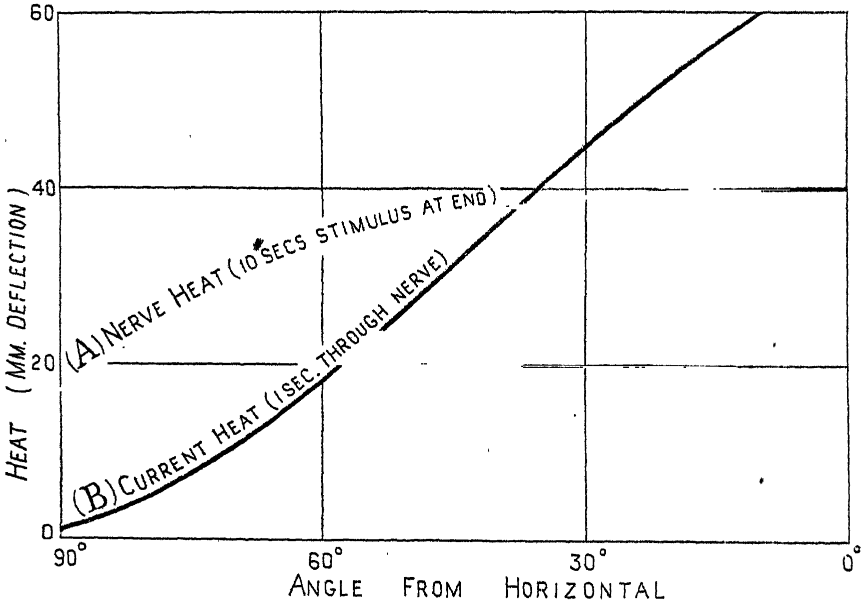

Fig. 4.—Relation between heat produced and angle of elevation of secondary of stimulating coil. Ordinates in mm. deflection, corresponding to initial heat in the case of the stimulus, and to total heat in the case of the warming. Abscissæ, degrees of elevation. (A)—Heat liberated by nerve in response to stimulus at electrodes 1 and 2 (see fig. 3). (B)—Heating effect of stimulating current measured by hot-wire.

as the square of the cosine of the angle of the coil. If the observed heat from stimulating the live nerve were a physical effect of the current, it should show a similar relation, whereas, in fact, it starts at high level with very weak stimuli, and soon reaches a maximum, beyond which no degree of stimulation will drive it—a further confirmation of the all-or-none law for nerve. (See fig. 4.) In the regular experiments just-maximal stimuli were used.

It is obvious, therefore, that with suitable precautions one is able to isolate the heat due to the physiological activity of the nerve, free from any error due to heat liberated by the stimulus itself. It is well, however, to emphasise the absolute necessity of such precautions.

(2) *Time Relations of the Heat-production.*—Following an "instantaneous" (1 sec.) electrical heating of the nerve on the thermopile, the galvanometer reaches a maximum deflection in about 8·5 secs., and returns to zero in less than 90. The lag on the upstroke is due mainly to the galvanometer system, and could be reduced by employing more rapidly moving galvanometers; that on the return is due to the heat equilibration within the thermopile,

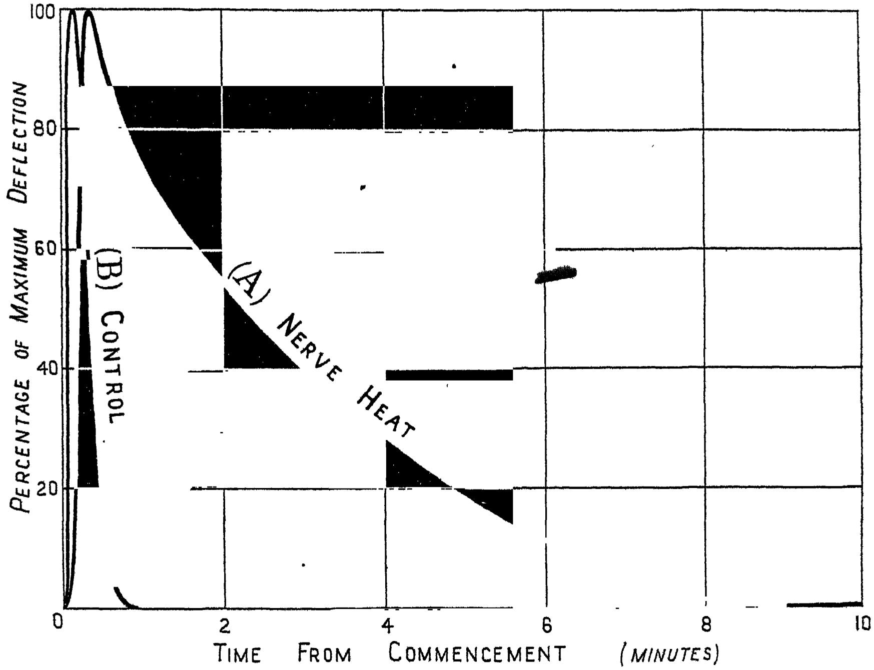

Fig. 5.—Vertically deflection of galvanometer as percentage of maximum: horizontally time in minutes: (A), in the case of heat liberated by nerve after stimulus commencing at time zero: (B), in the case of nerve warmed at time zero by current passed through its length.

which is rapid because of the small mass of nerve employed. When in a live nerve heat is produced by stimulation the galvanometer reaches its maximum, as before, in about 8·5 seconds from the end of stimulation, but then, instead of rapidly falling back to zero, it maintains for many minutes a slowly-decreasing deflection. Figs. 5 and 6 represent, on a small and a large scale respectively, the differences between these deflections.

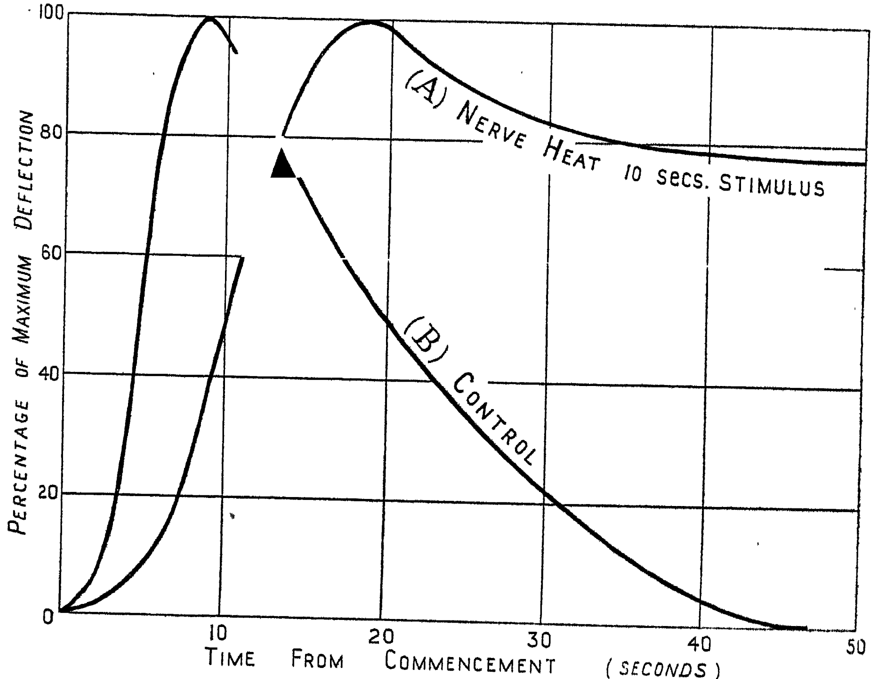

Fig. 6.—Same as fig. 5, but drawn on a different scale: vertically, deflection of galvanometer as percentage of maximum; horizontally, time in seconds. (A), nerve heat. (B), control. (A) represents the average of 15 curves from several experiments, 10 secs. stimulus at end of nerve. (B) represents the average of 25 curves.

A live nerve at 15° C. continues to produce heat at a gradually decreasing rate for about 10 minutes, though the exact point at which the curve reaches the base line is naturally somewhat uncertain. The area (A_l) of the deflection-time curve of nerve-heat varies as widely as does the initial deflection (D_l) in different experiments, but the ratio A_l/D_l is fairly constant, being for a 10-seconds stimulus about 190. Since in control experiments A_c/D_c is about 20, the live nerve produces almost ten times as much heat for a given maximum deflection as does a control heating of short duration.

For a more accurate study of the evolution of heat the curves may be analysed by the method described by Hartree and Hill (15). The results of a comparatively rough analysis on these lines, averaged for 4-second intervals, are shown in fig. 7. These confirm the idea of a separation of the heat-production into two phases, but are hardly accurate enough so far to do more. There is not the sharp division into an initial heat, intense but brief, and a recovery heat, small but prolonged, that is typical of muscle. If the initial heat exists as a separate entity, it is small when compared with the recovery heat, which follows very rapidly upon it. It is hoped later, by quickening the galvanometer response, to obtain a more decisive analysis.

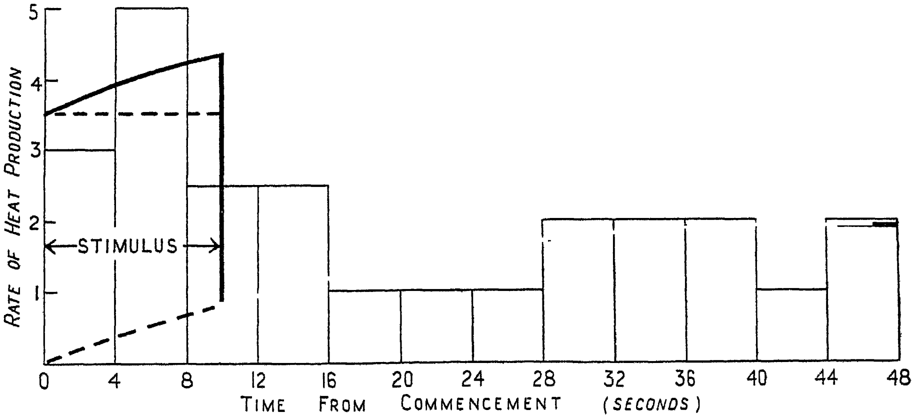

FIG. 7.—Approximate analysis of the nerve-heat deflection of fig. 6. Vertically, rate of heat production in arbitrary units: horizontally, time in seconds from commencement of stimulus.

Discussion.

It is not our purpose in the present paper to enter very closely into theories of nervous conduction. Since the heat produced by activity in nerve can now be measured, another method of study has been added to the response resulting from it and the action current. This method, however, has hardly begun to be exploited as yet, and since preliminary observations involving variables other than length of stimulation have given rather unexpected results, we prefer to leave a fuller discussion for future communications. Certain conclusions, however, follow from the absolute quantity of heat found, and its distribution in time relative to the stimulus, and it may be of interest to indicate these.

1. *Relation to CO_2 output.*—The most recent, and apparently most reliable, work on the CO_2 output is that of Parker (6), who reports a resting production

of CO_2 by frog's sciatic nerve of 0·0089 mgms., or 0·0044 cc. per grm. per minute, and a 14 per cent. increase in this amount on stimulation, or 0·0006 cc. per grm. per minute of stimulation. If we assume that the basic chemical reaction in nerve is the oxidation of carbohydrate, the CO_2-equivalent of the heat produced would amount to about 0·0008 cc. per grm. per minute of stimulation, a remarkably close agreement, giving some degree of probability to the assumption.

2. *Relation to oxygen consumption.*—No satisfactory data on the increased consumption of oxygen by active nerve are available, though Adam (4) and Haberlandt (3) agree in taking the *resting* oxygen consumption at about 0·001 cc. per grm. per minute, about twice Thunberg's value (26). If carbohydrate does furnish the energy for nervous activity, the respiratory quotient should be unity and an extra 0·0006 or 0·0008 cc. of oxygen be consumed per grm. per minute of stimulation. Adam, though avoiding a definite figure, places the maximum increase due to activity at 0·0013 cc.

A curious discrepancy appears between the resting values of the oxygen consumption and the CO_2 evolution, as reported by Adam and Haberlandt on the one hand, and by Parker and Tashiro on the other. The two former agree in placing the resting oxygen consumption at about 0·001 cc. per grm. per minute, while the two latter give the resting CO_2 production as about 0·004 cc. The errors of determination are sufficient to prohibit too serious a discussion of the resulting respiratory quotient of 4. Thunberg's results, giving a respiratory quotient of about 1, would appear, in this respect, more likely.

The solubility of oxygen in water, at 15° C. and 760 mm. pressure, is 0·034 cc. per cubic centimetre of water. Exposed to air water would dissolve 0·007 cc. per cubic centimetre, and for nerve exposed to air we may assume a value for the dissolved oxygen of 0·005 cc. per grm. If 0·0008 cc. be used per minute during activity by 1 grm. of nerve, the oxygen dissolved in it when in equilibrium with air, should last for 6 or 7 minutes of continuous stimulation. This figure neglects, however, the oxygen used at rest, to which that of activity is an addition. If 0·001 cc. be used per minute by 1 grm. of resting nerve (Adam) the dissolved oxygen would last the resting nerve about 5 minutes and the stimulated nerve about 3 minutes; if 0·004 cc. be used (the equivalent of 0·004 cc. of CO_2 given off—Parker) the dissolved oxygen would last the resting nerve $1\frac{1}{4}$ minutes, the active nerve only 1 minute. Since nerve trunks are supplied with relatively little blood they have no access to a plentiful supply of oxygen, and these considerations might, in a rough way, apply in the intact animal—in which case a resting nerve would soon asphyxiate. Even Thunberg's values would allow the resting nerve removed from air to nitrogen only about 12 minutes' supply of oxygen, or, if stimulated, only 4 minutes' supply.

There is clearly something anomalous in these results which should be explained. Moore (22) also has studied the CO_2 production of resting peripheral nerve, brain and muscle. Although no absolute values are given, he states that the nerve gave out only 10 to 30 per cent. as much as muscle or brain tissue. According to Meyerhof (23) a resting muscle consumes about 0·0003 c.c. of oxygen per grm. per minute, so from Moore's data a nerve would use not more than 0·0001 c.c. per grm. per minute, and the dissolved oxygen would supply a nerve for nearly an hour during rest. It is difficult to believe that resting nerve fibres would use so much more oxygen than a resting muscle, as a comparison of the results of Parker and Tashiro on the one hand, and of Meyerhof on the other, would suggest. It would seem much more likely that the calculation from Moore's and Meyerhof's data more truly represents the facts. It is clear in any case, assuming the heat observed to come from the oxidation of some ordinary foodstuff, in which case 1 cc. of oxygen must be the equivalent of about 5 calories, that the oxygen dissolved in a nerve exposed to and in equilibrium with air, would not last for more than 6 or 7 minutes of continuous stimulation without a further supply by diffusion.

Much work was done, 15 to 25 years ago, on the fatigue produced by stimulation in nerves, especially when deprived of oxygen. [*See*, for example, Fröhlich (27), Fillié (28), Thörner (29), Haberlandt (30), Tigerstedt (31).] The results of the present heat-measurements have an obvious bearing on the fatigue and recovery studied in those investigations.

3. *Stored carbohydrate.*—If glycogen be the ultimate source of energy for the nerve impulse, only a minute amount of it would be required for prolonged activity; only about 0·0006 grm. of glycogen per grm. of nerve, or 0·06 per cent., would be oxidised in ten hours of continuous stimulation (neglecting the resting metabolism).

4. *A comparison with muscle.*—In muscle the energy of the initial process is derived from the breakdown of glycogen into lactate, while in recovery it is derived from the oxidation of about one-quarter or one-fifth of the lactate originally formed, accompanied by the reinstatement of the rest as glycogen. The recovery heat is about 1½ times the initial heat. In nerve the recovery heat, according to the comparatively rough estimate we have been able so far to make, is about eight times the initial heat. This would seem to prohibit any possibility of the initial process which occurs during stimulation of the nerve being due to the formation of lactate from glycogen, followed by a

reinstatement of the major portion of the lactate to glycogen during recovery.

5. *Comparison with energy of action current.*—The heat liberated per impulse cannot be stated with any assurance, for observations so far made render it doubtful if the heat varies directly as the frequency of stimulation. With a coil providing 280 shocks per second the heat per impulse has the following values: initial heat 3×10^{-8} cal. per grm., total heat $2 \cdot 5 \times 10^{-7}$ cal. per grm. The energy of a single action current traversing a gram of nerve has been calculated on certain assumptions as $3 \cdot 5 \times 10^{-10}$ cal. (Hill (20)). The order of magnitude of these quantities must be essentially correct, whence it follows that far from representing the entire process in a nerve impulse, the action current is, from the energy standpoint, only a very small fraction of it, between 0·1 and 1 per cent.

6. *Energy per fibre.*—The number of fibres in our nerves was not determined, but accurate counts of similar specimens are available and may be used. Dunn (21) reports an average of 3,500 fibres in the frog's sciatic below its branches to the thigh, and we may assume this number for our nerves, eight of which, therefore, contained 28,000 fibres. The initial heat produced by this part of the nerve, obtained by multiplying the heat per grm. by the mass, is about $4 \cdot 5 \times 10^{-7}$ cal. per sec. of stimulation, while the total heat is about 4×10^{-6} cal. per sec. Taking the latter value, each fibre, for a 2 cm. length, would give $1 \cdot 4 \times 10^{-10}$ cal., or 7×10^{-11} cal. per cm. per second. This represents the total heat, the major portion of which is liberated after the impulses have gone by.

Assuming a frequency of 280 impulses per second, the heat produced in a centimetre length of one nerve fibre by a single impulse amounts to $2 \cdot 5 \times 10^{-13}$ cal., or about 10^{-5} erg.

7. *Bearings on the mechanism of the nervous impulse.*—If we are to accept a picture of the nervous impulse based upon Lillie's (25) iron-wire model, it is necessary to suppose that the reaction underlying the impulses takes place over the whole surface of the nerve fibre. It is interesting to calculate, on the assumption that the whole surface of a fibre is involved, the amount of energy liberated by 1 grm. molecule of the substance reacting in the surface.

Assuming, as Parker found, that half the volume of a nerve is occupied by conducting elements and that these have an average diameter of 6μ, it can be calculated that the area of the nerve fibres contained in 1 grm. of nerve is about 1,500 sq. cms. This cannot pretend to be an accurate estimate, but it is certainly of the right order of quantities for the area of the outer boundary of the nerve sheath. The area of the axis cylinder must be appreciably smaller. If now we assume that the surface of a nerve fibre is covered with a monomolecular film of some substance which prevents reaction between the inside and outside, or in some other way maintains a state of polarisation, we may calculate the area occupied by 1 grm. molecule of such substance, assuming it to be not dissimilar in its general nature from a fatty acid. According to Adam and Langmuir the area per molecule of a condensed film of such substance is about 25×10^{-16} sq. cm., so that 1 grm. molecule containing 6×10^{23} molecules would occupy $1 \cdot 5 \times 10^{9}$ sq. cms. Thus 1 grm. of nerve would contain on the surface of its fibres about one millionth of 1 grm. molcule of material. This again cannot pretend to be an exact estimate, but it certainly is of the right order of quantities.

The initial heat per impulse per grm. of nerve is about 3×10^{-8} cal., which, reckoned per grm. molecule of substance in the surface of the nerve fibres, is about 0·03 cal. Even if we take the total heat produced by the impulse it will amount only to about 0·3 cal. per grm. molecule. Compared with the heat of reaction of any ordinary chemical process this value is extremely small; for example, the heat of neutralisation of 1 grm. molecule of acid by alkali is about 13,000 calories; the heat of oxidation of 1 grm. molecule of lactic acid, about 144,000 calories. It is clear, therefore, either (*a*) that if the reaction involved in a nerve impulse takes place over the whole surface of the fibre, as presumably it would do on the analogy of the iron-wire model, then the complete reaction* involved is, from the energy standpoint, an extremely insignificant one; or (*b*) that the process of transmission does *not* involve the whole surface of the nerve fibre, but only a small proportion of it.

Summary.

1. It has proved possible to measure the heat produced by an isolated frog's nerve undergoing a faradic stimulus located at a point distant from the temperature-recording apparatus. Controls have shown that, with suitable precautions, no error is introduced by the heat liberated by the stimulus itself.

2. A detailed account is given of the methods used. The essential features are: (*a*) the employment of a thermopile with a large number of junctions, (*b*) the use of thermal amplification of the galvanometer's movements,

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* This does not eliminate the rather remote possibility that reactions of considerably greater energy may proceed during the rising phase, and be completely reversed during the falling phase, of the propagated impulse.

(*c*) adequate electrical insulation, (*d*) prolonged thermal equalisation, (*e*) calibration of the apparatus in absolute units, (*f*) the study of the heat after, as well as during, the stimulus.

3. About 90 per cent. of the total heat is liberated after the stimulus is over. A rough analysis of the results indicates a small initial heat production, followed by a prolonged phase of heat production lasting for 9 to 11 minutes.

4. The heat liberated in the initial phase averages about $7\cdot6 \times 10^{-6}$ cal. per grm. of nerve per sec. of stimulation; the total heat about $6\cdot9 \times 10^{-5}$ cal. per grm. of nerve per sec. of stimulation.

5. Previous experiments tended to indicate that the heat-production, if any, was much smaller than this. The reasons for the discrepancy are (*a*) the absence of a direct calibration in the earlier experiments, and (*b*) the lack of realisation that a large fraction of the heat might appear in a recovery phase.

6. The absolute values obtained are compared with the results of other observers on the oxygen consumed and the CO_2 liberated owing to nervous activity. In particular, the observations of Parker on the extra CO_2 output agree well with the total heat observed, on the hypothesis of the oxidation of some ordinary foodstuff.

7. The possibility of measuring the heat-production of stimulated nerve obviously opens up new avenues for the study of nerve function. The matter is being pursued, and only a few deductions from the absolute values are given here.

The expenses of this research have been defrayed by grants from the Royal Society. We are indebted to the Department of Scientific and Industrial Research for a personal grant to one of us (A.C.D.).

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Responses to Rhythmical Stimulation of the Cerebral Cortex.—Preliminary Communication.

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(Communicated by Sir Charles Sherrington, O.M., F.R.S.—Received June 10, 1926.)

(From the Physiological Laboratory, Oxford.)

[PLATES 6 and 7.]

François-Franck and Pitres (1) in 1878–79 showed, by graphic analysis of movement produced by stimulation of the cortex cerebri, that complete fusion of responses did not occur until the frequency of the stimulation was increased above 45 per second. With the same apparatus they found that a muscle tetanus showed the stimulus rhythm up to the same frequency, and no further. Horsley and Schafer (2) in 1886, after a number of experiments on both voluntary contraction and movement by cortical stimulation, concluded that:

"The rate of the rhythm is not the same as that of the excitation except when the frequency of excitation is 10 per second or less. With all higher rates of excitation the rhythm of muscular response is maintained at a fairly uniform rate of about 10 per second" (p. 105). Horsley and Schafer discuss the results of François-Franck and Pitres, quoting a statement from which they conclude that these authors modify their former conclusions; Luciani (3) also refers to this so-called modification. We find, however, on referring to François-Franck and Pitres' paper (4), that their statement is to the effect that the *regularity* of cortical myograms is not as complete as in muscle nerve tetanus. They do not modify their original assertion that a stimulus rhythm of up to 45 per second to the cortex can appear in the muscle response.

It seemed advisable to repeat these experiments, using an accurate torsion-wire myograph. The electromyogram has been recorded simultaneously; as far as we can ascertain this is the first attempt at the analysis of the action-currents due to cortical stimulation.* The results obtained from the first three experiments are of some interest, therefore this preliminary communication is being made; further experiments are being carried out.

Method of Experiment.

Three monkeys were used in the investigations, a *Macacus Rhesus*, a Callothrix and a *Cercopithecus Mona*. The motor cortex was exposed under ether anæsthesia and muscles of the opposite forelimb were prepared for attachment to the myograph. The muscles used were brachialis anticus and biceps brachii in the first animal, brachialis anticus and short heads of triceps in the second, and extensor carpi radialis, flexor carpi radialis and supinator longus in the third. The remainder of the limb was immobilised by nerve or tendon section. The fixation of the muscle attachments was by heavy drills clamped to the table, and a clamp to the blade of the scapula in the case of the biceps.

The mechanical records were obtained by attaching two of the muscles, by means of wire hooks, to a double torsion wire myograph. This recorded in the same optical system as the fibre of a string galvanometer of the Cambridge pattern. The natural frequency of each wire of the myograph is over 1,000 per second, and loaded with a hook it is 970 per second. The muscle records isometrically. The stimulation was by means of a Sherrington torsion wire key delivering a regular series of break shocks. In these experiments, rates of 18 to 68 stimuli per second were used. A unipolar stimulation was applied, the indifferent electrode being attached to the ipsilateral hind limb.

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* Since this paper has gone to press it has been brought to our notice that P. Hoffmann, ('Archiv f. Anat. u. Physiol.,' *Physiol. Suppl.-Band*, 1911, p. 286), stimulated the cerebral cortex of the dog and recorded the action currents from m. sartorius. He found that a stimulus rate of 50 per second produced a total electromyographic rate of 50 per second, and with higher and lower rates of stimulation the total currents numbered about 60 per second.

I. *General Form of Mechanical Response.*—The *ascending phase* of the myographic response always shows recruitment (fig. 1) as opposed to the reaction d'emblée type of response usually seen in reflex flexion (5). The rate of recruitment seems to bear no relation to the point stimulated, but within two seconds of a previous stimulation of the same point the rate is facilitated. This is seen equally well in flexors and extensors.

The *plateau* may be even or irregular, but it is generally more even at slower rates of stimulation. Some of the responses are clonic in form.

The responses show practically no *plateau after-discharge*, the plateau being maintained usually for between 40 σ and 80 σ (longest time 120 σ) after the last stimulus in the flexor brachialis anticus, as compared with 40 σ in a flexion reflex obtained by stimulation of the ipsilateral ulnar nerve, and with 35 σ in a motor nerve tetanus, both obtained in the same muscle under approximately the same conditions of anæsthesia. In the extensors the plateau after-discharge is sometimes slightly longer, but is usually 20 σ to 60 σ as compared with 30 σ in the motor nerve tetanus in the same muscle under anæsthesia. Clonus in the plateau does not prolong the after-discharge. Occasionally, after a small response, a terminal rebound phenomenon is seen; this is an indication of concurrent inhibition.

The *relaxation* shows some after-discharge, but this is usually slight and of the same order as in a spinal flexion reflex. Clonus in the after-discharge is usually preceded by complete relaxation.

II. *Electric Responses.*—The action currents were all obtained from the brachialis anticus. When attempts were made to lead off from the extensors (triceps or ext. carp. radialis) it was found difficult to eliminate escape of the stimulating current to the proximal galvanometer lead. But with the flexor muscles, it was proved that the string movements were caused by true electric responses. The action currents show a marked resemblance to those found by Cooper and Adrian (6) for limb reflexes in the decerebrate cat. The rates of stimulation were only varied between 68 and 18 per second, and at these frequencies it is generally possible to trace the stimulus rhythm through the record; it is interesting to note that this is most marked when the mechanical record shows the stimulus rhythm as well. Single responses at these frequencies are comparatively rare, each primary wave being generally followed by a varying number of secondary waves. At the low rates of stimulation there may be three or four secondary waves to each stimulus. In some records the secondary waves increase in size until they are indistinguishable from the primary waves; the result may be an irregular rhythm, varying between 100 and 150 per second; or more often there is a regular rhythm at double the rate of stimulation. Examples of the different rates are given in the Table.

Electric responses in brachialis anticus.

Expt.	Stimulus frequency per second.	Large waves per second.	Total waves per second.
1. Macacus Rhesus	50	50	150
	50	50, 25	100, 150
	50	not marked	100, 150
2. Callothrix.	68	136	136
	68	68	136
	68	68, 34	136
	60	60	120
	48	irregular	110
	48	48	96
	48	48	96, 144
	48	irregular	96–120
	36	36	108, 144
	18	18	72, 90, 108

The rate in the after-discharge has been irregular and varies between 90 and 150 per second. In two cases where there is clonus after the stimulus there was a regular rhythm of 130 per second. In no case has a total rhythm of over 200 per second been recorded, and it will be interesting to note in later experiments whether the upper limit for regular action currents from cortical stimulation agrees with this. Two records were obtained from the Callothrix of a flexor rigidity when the anæsthesia was getting rather light; these show distinct rhythms of about 110 per second. This monkey did not show rhythms greater than 150 per second, and the average figure for the total waves was about 120 per second, which agrees with the figure for the rigidity. The first monkey (*Macacus Rhesus*) seemed to have a slightly higher figure, *i.e.*, 150 per second. The third monkey (*C. Mona*) was not in very good condition and the electric responses were poor.

In the ipsilateral flexion under the same conditions the primary waves follow the rate of stimulation of 68 per second; each response usually shows one secondary wave, thus giving a total rate of 136 per second.

III. *Rhythm of Mechanical Response.*—The highest rate of stimulation used was 68 per second, and out of ten records of the brachialis anticus at this frequency, five show places where this rhythm is present for a few responses. These responses are usually seen on the ascent plateau turn, but are also found on the plateau, though rarely for more than one or two responses. The triceps shows traces of this rhythm in two records, and the ext. carp. radialis shows distinct separate responses to a rate of 64 per second stimulation, *e.g.*, fig. 3, where the responses show as a minute rippling on the mechanical record.

Motor nerve tetani of both the brachialis anticus and the triceps used for the cortical observations, under anæsthesia at 68 per second, show well-marked stimulation rhythms in the mechanical records.

At 60 per second, rhythmic responses of frequency corresponding with that of the stimulus rhythm become more obvious and maintained in the mechanical records both of flexors and extensors. At rates lower than this, *e.g.*, 50 per second, nearly every record shows some trace of the stimulating rhythm, and in many records, *e.g.*, 36 per second, the rhythm can be followed throughout the time of stimulation in both extensors and flexors.

The stimulus rhythm in the mechanical record showed a tendency to run into groups corresponding to varying numbers of stimuli. Even when the stimulus rhythm is very marked, there is a tendency for a deeper notch after each alternate response, as is often obtained in spinal reflexes. At a high rate of stimulation this grouping in pairs gives the appearance of a mechanical rhythm at half the stimulation rate. This paired rhythm is often interrupted by groups of responses corresponding to greater numbers of stimuli. In antagonistic muscles this grouping may be concurrent in the two muscles, but sometimes it is alternating, *e.g.*, fig. 5.

The plateau of the response usually shows a tendency to become clonic, *e.g.*, fig. 7. The rate of this clonus during stimulation varies from 20 to 4 beats per second. Clonic after-discharge, which was elicited by strong or prolonged stimulation, had the same time relations, but did not necessarily follow, or preclude, clonus during the stimulation. The rising phase of a fully developed clonus shows the rhythm of stimulation, while the falling phase shows the paired grouping described above.

Summary.

The electromyogram showed consistently, up to the highest rate of stimulation employed (68 per second), primary waves agreeing in frequency with the rate of the electrical stimuli applied to the motor cortex. The primary waves are usually accompanied by secondary waves, giving an average total frequency of 120 responses per second.

Rhythmic tremor corresponding in frequency with the rates of electrical stimuli applied to the cerebral cortex appeared for short periods in the mechanical records of the contracting muscles, even for the highest rates used (68 per second). For rates of cortical stimulation slower than 68 per second, *e.g.*, 50 per second, the muscular contraction showed rhythms corresponding with the cortical stimulus more obviously and persistently. This confirms, and extends to somewhat higher frequencies, the original observations of François-Franck and Pitres.

Muscular contractions evoked from the motor cortex were characterised by a slow recruitment, even in the case of flexor muscles. Relatively little after-discharge was met with in both the extensors and flexors. Slower clonic variations of tension at an average rate of 10 per second were observed frequently and probably accounted for the results of Horsley and Schafer.

The transmission of such high rates of stimulation from the motor cortex to the responding muscle, and the small amount of after-discharge, together with the prominence of the primary waves in the electromyogram, indicate a much more direct connection with the spinal motor neurone than might have been expected.

We wish to acknowledge our thanks to Sir Charles Sherrington for his kindly advice and criticism.

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DESCRIPTION OF PLATES 6 and 7.

Simultaneous electrical and mechanical records of contractions elicited by unipolar stimulation of the motor cortex in the monkey. Time is shown by small verticals at 0·02 second. The interrupted line at the bottom shows the rate of break shock stimuli from a coreless Berne induction coil. Above this are the signals showing the beginning and end of the stimulation; all the records read from left to right. The line of zero tension is shown above the signal lines. The myograph to which the flexor muscle is attached has a fine hair fixed near the point and thus traces a double line in each case. The tension recorded by the myograph is indicated on the left border of fig. 4, and this scale applies to all records except in fig. 7, which has its own scale attached. String resistance 2,100 ohms; tension 5 mm. for 1 mv. except in fig. 7. The galvanometer leads (AgCl type) are in the belly and tendon of the muscles.

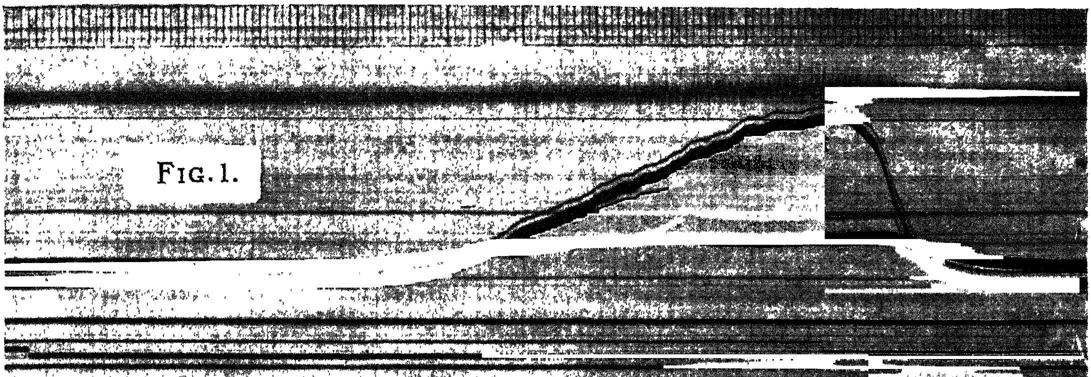

FIG. 1.

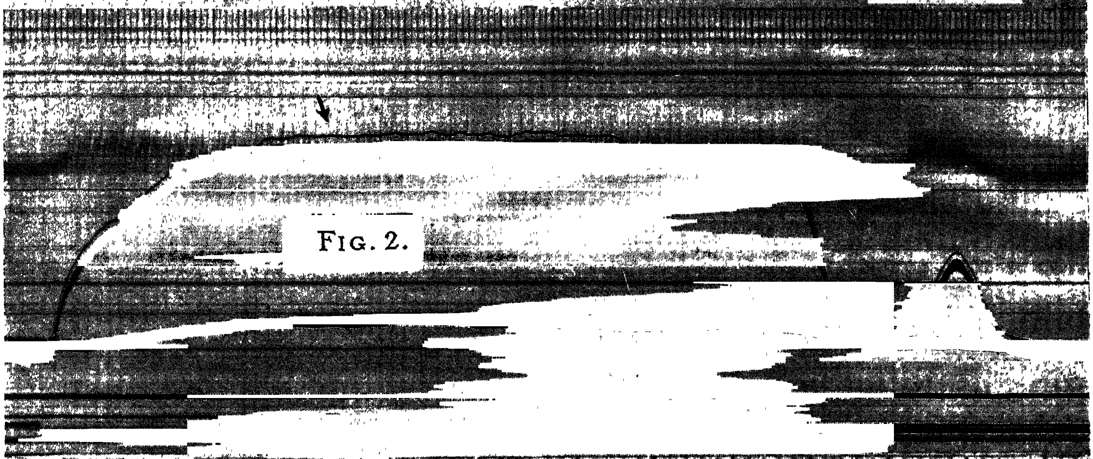

FIG. 2.

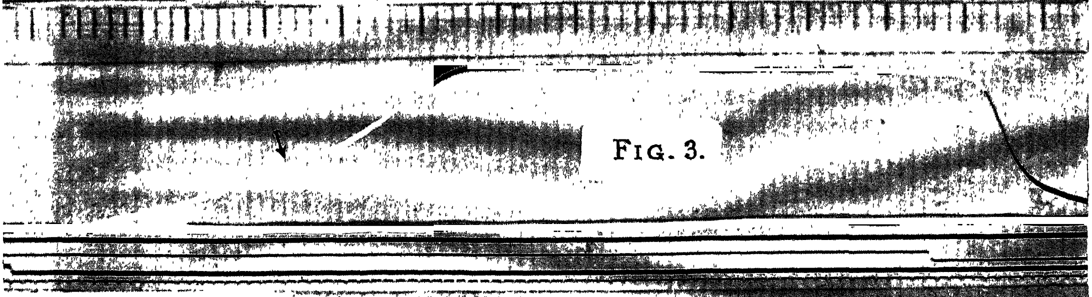

FIG. 3.

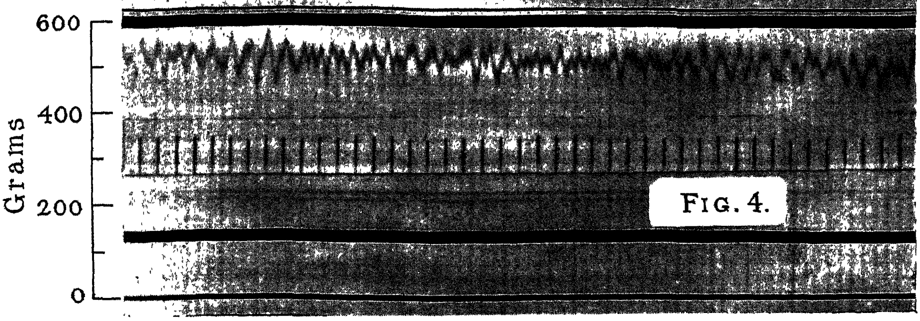

FIG. 4.

FIG. 5.

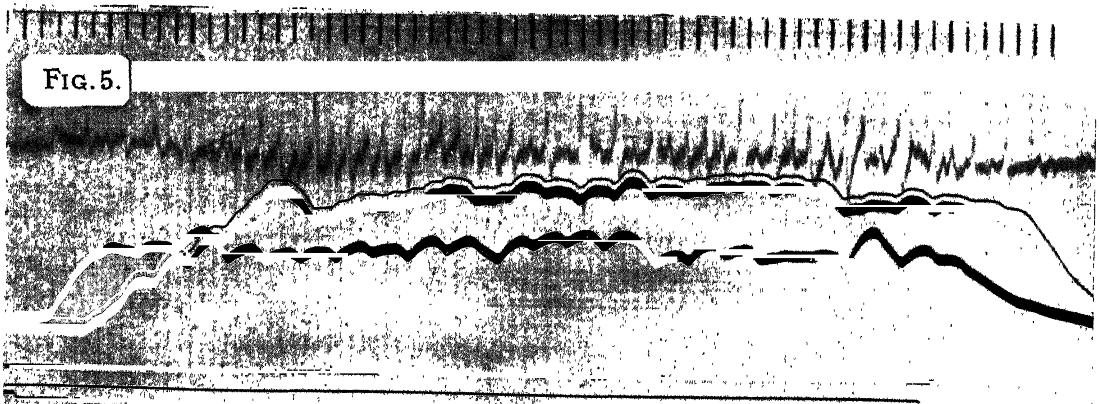

FIG. 6.

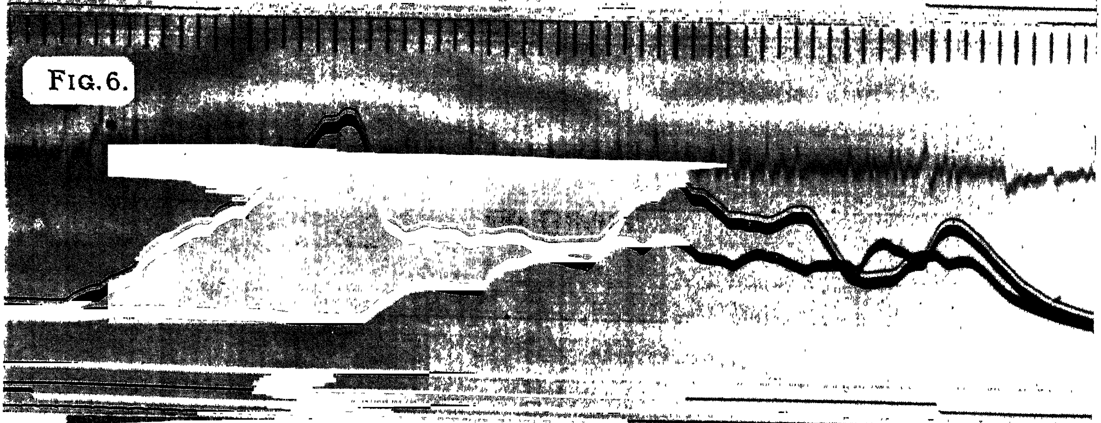

FIG. 7.

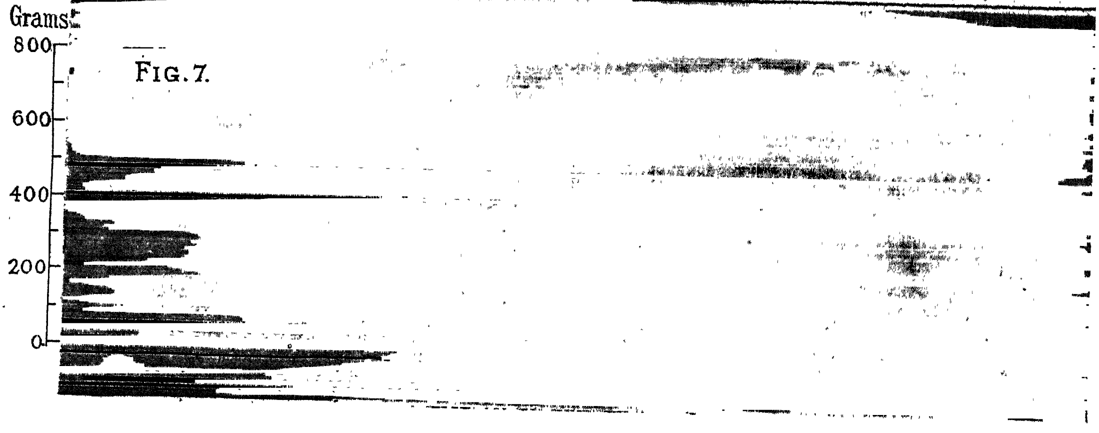

PLATE 6.

FIG. 1.—Callothrix. Brachialis anticus (flexor) and triceps (extensor). Galvanometer leads in brachialis. Rate of stimulation 60 break shocks per second. Coil distance 6 cm.

Note.—Recruitment, transmission of full stimulus rhythm at place indicated, slight after-discharge, and primary waves in the action currents becoming more prominent as stimulation proceeds.

FIG. 2.—Muscles and leads as in fig. 1. Bk. sks. at 60 per sec. and 6 cm. coil distance. Response facilitated by previous stimulation.

Note.—Recruitment facilitated, full stimulus rhythm at place indicated, well-marked primary waves all through electric response and first contraction of clonic epileptiform after-discharge.

FIG. 3.—C. Mona. Extensor carpi radialis. Bk. sks. at 64 per sec. and 4 cm. coil distance.

Note.—Recruitment, even plateau, little after-discharge and stimulus rhythm indicated on ascent.

FIG. 4.—Callothrix. Rigidity of brachialis anticus during a light stage of anæsthesia.

Note.—Electric responses showing a rate of 120 per sec.

PLATE 7.

FIG. 5.—Callothrix. Brachialis and triceps. Bk. sks. at 48 per sec. and 6 cm. coil distance. Leads from brachialis.

Note.—Concurrent response of flexors and extensors, rhythm of stimulation in flexors, marked pairing in both muscles, grouping of responses alternating in the two muscles, and marked primary waves showing a relation to the mechanical rhythm.

FIG. 6.—Callothrix. Muscles and stimulus as in fig. 5.

Note.—Late response of triceps accompanied by relaxation of brachialis, and disappearance of primary waves during this relaxation and also during later relaxation.

FIG. 7.—Macacus Rhesus. Brachialis anticus (double record and electric responses) and biceps brachii (single record). Bk. sks. at 50 per sec. and 6·8 cm. coil distance. String tension 7 mm. for 1 mv.

Note.—Long latent period and clonic nature of response. Stimulus rhythm on ascending phase of each clonic discharge. Primary waves in the action current at the start are at half the rate of stimulation, later they follow, and at the end the large waves are at double the stimulation rate.

Observations on Concurrent Contraction of Flexor Muscles in the Flexion Reflex.

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(Received June 16, 1926.)

[PLATE 8.]

The "flexion reflex," flexing the limb (hind) at hip, knee and ankle, is elicitable from any one of a number of the various afferent nerves of the limb. It reveals to inspection little difference whichever be the particular afferent nerve stimulated. That the flexion evoked cannot, however, be strictly the same under excitation of the several different nerves has been already shown (5). To examine further the differences it thus presents as evoked from the several nerves we have taken simultaneous myograms from paired flexor muscles under provocation of the reflex from various different afferent limb-nerves. The muscle pairs selected have been (1) a hip-flexor and a knee-flexor (tensor fasciæ femoris and semitendinosus) together, (2) a hip-flexor and an ankle-flexor (tensor fasciæ femoris and tibialis anticus) together, and (3) a knee-flexor and an ankle-flexor (semitendinosus and tibialis anticus) together.

I.—*Method.*

The preparation (cat) has, after spinal transection in the anterior lumbar region and immediately subsequent decerebration under deep anæsthesia, been made ready for the myograph by appropriate isolation of the test muscles. The method of fixation and attachment to the myograph has been as described in previous communications, except that for tibialis anticus the freed tendon has been passed round a small light pulley allowing in its case a horizontal pull on the myograph. The resting tension of the two muscles has been adjusted for near equality. In using tensor f. femoris for the myograph distinction was made between its long anterior portion, tens. f. fem. longus, and its short posterior portion, tens. f. fem. brevis. Most of the observations with it were made on the former, the tens. f. f. brevis being cut away, though not always to equal extent. The myograph employed is of isometric pattern and records optically: its description is given as an Appendix to this paper; it is a further modification of the myograph described in a previous communication (6). The afferent nerve, bared and cut, has been stimulated by single or double-shock series of various frequency delivered from a coreless coil fed by a 2-volt battery.

II.—*Results.*

There is, commonly, marked difference of strength between the contractions concurrently given by the pair of muscles, Plate 8, fig. 1. This might mean that of the two muscles one was a more powerful unit than the other, and that the afferent nerve reflexly activating them did so simply in the same proportion for both. The differences observed cannot, however, be fully accounted for in that way. The ratios between concurrent contractions of the same muscle-pair differ widely as yielded by different afferent nerves in one and the same preparation. Thus, in the same experiment and in almost immediately consecutive observations, hip-flexor and ankle-flexor being the muscle-pair employed and the following afferents stimulated successively at the same coil distance, internal saphenous nerve evoked from *tensor f. femoris* 660 gm. contraction concurrently with 950 gm. from *tibialis anticus*; popliteal nerve evoked from *tens f. f.* 190 gm. contraction concurrently with 1120 gm. from *tib. ant.*; external cutaneous nerve evoked from *tens. f. f.* 1510 gm. concurrently with 450 gm. from *tib. ant.*; and a digital branch of musculo-cutaneous nerve evoked from *tens. f. f.* 195 gm. contraction concurrently with 1370 gm. from *tib. ant.* The connection with a given flexor muscle is evidently different quantitatively for different individual afferent nerves of the limb (*cf.* text figure and fig. 1, Pl. 8).

Assessing the concurrent contractions by the tension-values developed in each, and stating that of the weaker as percentage of that of the stronger, instances of the ratios found, under stimulation not far from maximal for the afferent nerve, have been as follows :—

Afferent Nerve.	Hip-flexor. Tens. f. f.	Knee-flexor. Semitend.	Ankle-flexor. Tib. antic.
External cutaneous	100	23 (much	variation)
	100		30
		17	30
Internal saphenous	100	56	
	100		87
		55	87
Nerve to sartorius	100	37	
Superficial obturator	100	62	
		62	3
Nerve to quadriceps extensor	100		21
		13	21

Afferent Nerve.	Hip-flexor. Tens. f. f.	Knee-flexor. Semitend.	Ankle flexor. Tib. antic.
Popliteal nerve		42	100
	12		100
	3 (or less)	42	
Peroneal nerve	66	100	
Peroneal nerve distal to tib. ant.		100	69
	14		69
Digital branch of musculo-cutaneous	9		100
		93	100

It was noticed that fluctuations of reflex activity in one and the same experiment did not greatly disturb the ratio obtained between strengths of the reflex responses of the two test muscles. The ratios were, however, found to vary considerably from experiment to experiment. Thus, in one experiment, int. saph. nerve evoked 12 per cent. stronger contraction from semitend. than from tens. fasc. femoris. In regard to tensor fasciæ femoris this is traceable in part to the resection of the posterior short portion of the muscle in order to avoid that portion's lying slack under the initial tension applied in the resting position. The proportion of the muscle resection could not always have been the same. Variation of some of the ratios from experiment to experiment may in part be due to the well-known frequent segmental variation of the lumbo-sacral plexus from individual to individual. In one preparation hamstring nerve evoked no response from ten. f. f. longus (*cf.* 5), in three others it did evoke a weak response. Occasionally in one and the same experiment the ratio given under the same coil stimulus applied at different times to the same nerve varied considerably: thus digital branch of musculo-cutaneous yielded at one time semitendinosus contraction as 93 per cent. of tibialis anticus and at another 111 per cent.

One factor influencing the ratio was found to be the strength of the stimulus applied to the afferent nerve. With increase of the strength of the stimulus the strength of the reflex contraction commonly increases in both muscles but the increments do not maintain the same ratio. Thus: with internal saphenous nerve :—

Stim. in cm. on coil	22	20	18	16	14	13	12
Tib. antic. grm. tens.	90	225	290	310	750	860	860
Tens. fasc. fem. gm. tens. ..	380	610	700	620	900	980	1090

Coil in cm.	18	15	14
Semitend. gm. tens.	25	150	210
Tens. f. fem. gm. tens.	600	830	970

Coil in cm.	28	24	20	16
Semitend. gm. tens.	0	90	224	488
Tens. f. fem. gm. tens.	50	1265	1380	1760

Coil in cm.	25	24	22	20	18	16	14	13	12
Semitend. gm. tens	20	50	90	170	215	300	510	460	500
Tibial. ant. gm. tens.	30	110	240	390	480	630	750	730	750

Again, with nerve of quadriceps extensor muscle:—

Coil distance in cm.	20	18	16	14	13	12
Tib. antic. gm. tens.	30	60	75	170	230	360
Ten. f. fem. gms. tens.	250	660	820	1140	1330	1470

Again, with external cutaneous nerve of thigh:—

Coil dist. in cm.	25	24	23	21	20	19	18	16	14	13	12
Semit. gm. tens.	0	0	4	13	9	20	16	14	125	233	250
Tens. f. fem. gm. tens.	0	some	80	160	330	390	510	530	1530	1870	1980

In such instances the ratio of the less contracting muscle to the more contracting muscle somewhat steadily increases with increase of the stimulus strength. Where, however, the afferent is of more equal power upon the two paired muscles cases such as the following are met:—digital branch of musculo-cutaneous of peroneal:—

Coil in cm.	24	23	22	20	18	16	14	13
Tib. ant. gm. tens.	30	25	65	360	590	710	790	1040
Semitend. gm. tens.	35	60	90	330	560	630	860	1160

Here ankle-flexor along a certain range of stimulus gave the stronger contraction, although either side of that range it gave the weaker.

Again, with peroneal nerve:—

Coil in cm.	20	19	17	15	14	13	12
Tens. f. fem. gm. tens.	30	90	420	550	500	510	600
Semitend. gm. tens.	30	130	400	770	690	840	820

Coil in cm.	16·5	16	15	14·5	13·5
Tens. f. fem. brevis gm. tens.	35	200	320	390	490
Semitendinosus gm. tens.	60	200	400	580	1010

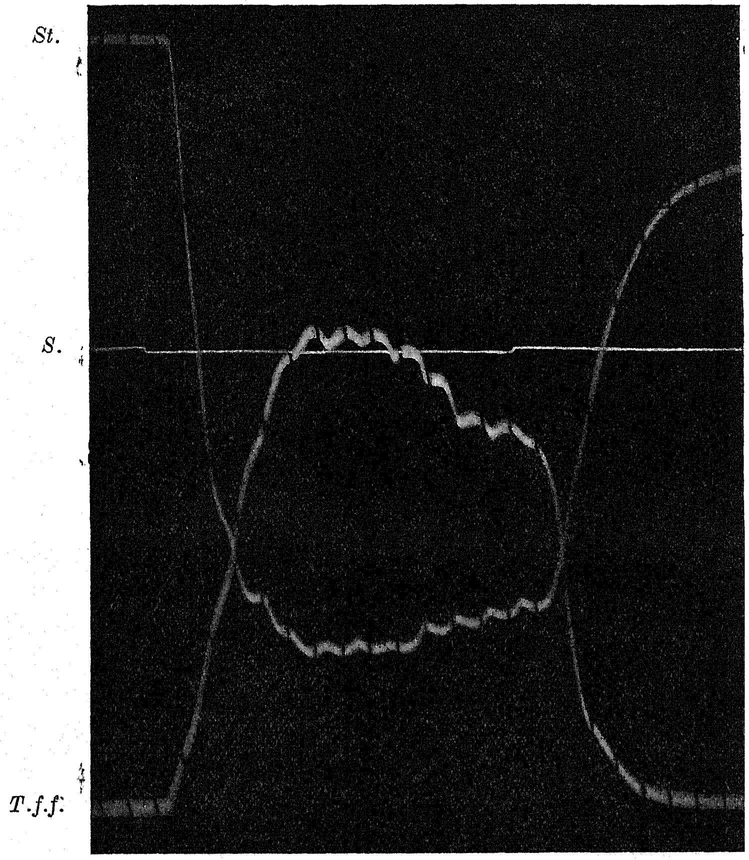
Similarly, in other instances, with peroneal nerve the increments in semitendinosus were relatively greater than in tens. f. f. with strong stimuli, and conversely with weaker.

That each increase of stimulus calls forth commonly an increment of contraction in both of the paired muscles indicates that among the motor units of each of those muscles are some which for the particular afferent nerve stimulated respond at like reflex threshold. We have in several instances sought to determine how far this equality of reflex threshold extends toward the limit of minimal stimulations of the afferent nerve. Naked eye inspection of bared muscles had already shown (5) that in this, the flexion-reflex, separate flexor muscles present differences of minimal threshold which are but slight under excitation from one and the same afferent nerve. The double myograph gave opportunity for better examination of this point in regard to the test muscles here taken, although the well-known variability of minimal reflexes presents difficulty.

Instances of comparison:—

Aff. nerve.	*Muscle pair.*	*Coil distance in cm.*	
Peroneal ..	Tens. f. fem. and semitend.	22 cm. neither	21 cm. both.
,, ..	,, ,, ,,	17 ,, ,,	16·5 ,, ,,
,, ..	,, ,, ,,	18·5 ,,	18 ,, ,,
Ext. cutan. ..	,, ,, ,,	25 cm. neither;	24, t. f. f. only, 23, both.
n. of sartorius..	,, ,, ,,	26 cm. neither	25 cm. both.
Int. saphenous	,, and tib. anticus	26 ,, ,,	25 ,, ,,
,, ,,	,, ,, ,,	23 ,, ,,	22 ,, ,,
n. of quadr. ext.	,, ,, ,,	22 ,, ,,	20 ,, ,,
Int. saphenous	tibialis ant. and semitend.	27 ,, ,,	26 ,, ,,
Digit. of musc. cutan.	,, ,, ,, ..	26 ,, ,,	25 ,, ,,

With internal saphenous in separate experiments the minimal reflex threshold for tibialis anticus was sensibly similar to that of tensor f. fem. on the one hand and of semitendinosus on the other, indicating that for this afferent the reflex minimal threshold was sensibly the same for hip-flexor, knee-flexor and ankle-flexor. For one and the same afferent nerve the inferior limit of its reflex threshold for concurrent contractions of each of the two test muscles has in the case of a number of the afferent nerves proved closely alike, perhaps identical. Yet for certain other afferent nerves that seemed not to be the case; thus, it was doubtful whether in the case of popliteal nerve its threshold of reflex action on tensor f. femoris is usually nearly so low as for semitendinosus; and other instances have met us.



Text-figure.—Concurrent reflex contraction of hip-flexor *T.f.f.* and knee-flexor *St.* in response to stimulation of ipsilateral peroneal nerve by single-shocks at 45 p.s. at 13 cm. coil distance. Tendon movement × 205 for each muscle: tension-development 200 grm. p. cm. ordinate; initial tension about 60 gm. for each muscle. *S*, shadow of short-circuiting key for stimulating circuit. Time in 1/50 sec.

The mechanical latency of concurrent contractions given by the paired muscles has not shown large difference as between the members of the pair. In some observations on hip-flexor and knee-flexor as test muscles the speed of the recording plate has allowed comparison of the latencies to an error of $\pm 1 \cdot 5 \sigma$. There, with internal saphenous as afferent, the greatest difference of latency between tensor f. fem. and semitendinosus was 3 σ, the hip-flexor being in every instance the earlier. With external cutaneous nerve the greatest difference was 6 σ, tensor f. fem. was always the earlier (Plate 8, figs. 1 and 2); with peroneal nerve the maximal difference was 4 σ and tensor f. fem. always the later (text-figure); with obturator nerve the greatest difference was 6 σ and tensor f. fem. always the earlier.

With hip-flexor and ankle-flexor as pair, the travelling surface used allowed readings not closer than $\pm 2 \cdot 5\ \sigma$; the response of tibialis anticus, the tendon of which acted round a light pulley, was with all the afferents found to be the later, but very rarely by more than 5 σ. When paired with semitendinosus it was also, with all the afferents tried, the later; but in three instances by less than 2 σ, subject to the latitude $\pm 2 \cdot 5\ \sigma$ of the reading.

That the above-instanced similarity both of reflex threshold and of latent period obtains for the concurrent contractions of the test muscle pairs amounts to the afferent nerve commonly bringing into play, with like facility and like time-relations, motor units widely scattered in the limb, irrespective of the circumstance that many of them lie grouped in separate and even distant individual muscles. From the point of view of the afferent nerve the muscular entity executing the reflex movement is in so far not this or that muscle but a composite aggregate of motor units, scattered through and forming parts of a number of separate and even distant muscles. This composite collection is given functional solidarity and homogeneity by the likeness of threshold and latency obtaining among its units in regard to the afferent nerve which brings them into action. An increment of stimulus does not break this homogeneity but merely brings to it a certain addition again composed of units mutually similar in threshold and latency although made up from muscles anatomically separate. The individual muscle, although an anatomical entity, does not of itself and as one whole offer such reflex unity as does the above anatomically heterogeneous group; nor does the individual muscle as such constitute a functional unit of the kind into which analysis of co-ordination resolves the motor apparatus executive of the reflex movement.

In the performance of a spinal reflex act therefore the executant muscular entity is not this or that individual muscle but a set of motor units made up from parts of various muscles. In carrying out this principle the several afferent nerves, although all severally evoking limb-flexion, combine the component part-contractions of the confederate muscles in proportions and degrees which are different and more or less characteristic for each individual afferent. The flexion-reflex is thus not wholly the same when evoked by different afferent nerves, even under comparable strengths of stimulus. The contraction of these or those particular flexor muscles is stressed according as the elicitation of the reflex is by this or that particular afferent nerve (5).

The term "flexion-reflex" therefore, just as the term "scratch-reflex," denotes, strictly speaking, a *group* of reflexes all more or less alike and all using approximately the same motor apparatus in approximately the same way, yet from one afferent to another differing in detailed distribution of the motor units employed, while yet always conforming to the general type "flexion-reflex" (7).

III.—*Other Features.*

Some features of these contractions concurrently provoked in the paired flexors seem of interest, aside from the main theme here dealt with. (1) The reflex after-discharge has been, with much regularity, despite variety of afferent nerve employed, more prolonged from semitendinosus and tibialis anticus than from tensor fasciæ femoris. In semitendinosus although prolonged it usually presents a sharp decline at outset. Where the after-discharge has taken the form of a stepped decline, we have observed very strict synchronism in the steps of the simultaneous records from the two muscles. (2) The rate of the stimulation has sometimes been low, *i.e.*, 45 single shocks a second; the reflex contraction of both muscles has then usually shown the stimulus rhythm with marked and about equal distinctness (see text-figure), but in some experiments one muscle and not the other has shown the stimulus rhythm. (3) Instances have occurred where the weaker contraction of a paired response has already begun to decline before the period of rise of the stronger fellow contraction has reached halfway. The weaker contraction has sometimes subsided altogether, despite continuance of the stimulation of the afferent nerve, at a time when the stronger contraction shows little or no decrease.

IV.—*Summary.*

The relative amount of contraction concurrently excited in pairs of muscles flexing hip, knee and ankle, under stimulation of this or that different afferent nerve, is examined and shown to be different and more or less characteristic for each several nerve.

Each afferent nerve possesses motor units, which in regard to itself are of practically similar threshold, scattered apart in separate and even widely distant muscles of the limb; and the latency of mechanical response of these is closely similar. The executant entity in the reflex is thus not this or that muscle but a composite collection of motor units, made up from portions of anatomically separate muscles.

With concurrent reflex contractions of two flexor muscles the after-discharge may, as between the two, differ considerably in amount although the stimulus for both is one and the same.

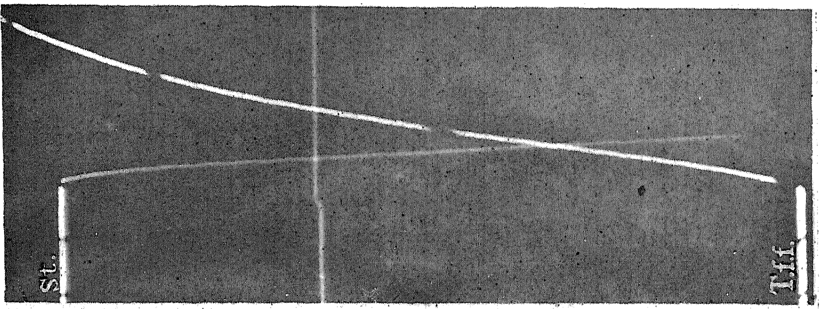
V.—*Appendix.*

By Sir Charles Sherrington, F.R.S.

The isometric myograph employed was a modification of that described in a previous communication (6). It has advantages of greater magnification of the muscle-shortening, and therefore closer approximation to pure isometric record and employment of smaller angular torque of the steel resistance. Moreover, the instrument provides simultaneous records from two muscles, against either equal or different resistances and under adjustable initial tensions.

The arrangement is as follows: The magnified image of an illuminated narrow rectilinear slit, similar in pattern to a spectrometer slit, is thrown through the horizontal slit-opening of an ordinary cardioelectrograph camera (Cambridge Scientific Instrument Co.) upon a falling photographic plate. The magnified image is obtained by a low-power microscope objective, in front of which, after the beam has been deflected by a prism, two little mirrors, one attached to each of the twin wires forming the double myograph, receive the image and reflect it separately to a second prism which deflects the thus paired images through a cylindrical lens condensing them upon the camera plate. Torsion of either wire displaces its member of the image pair horizontally on the vertically moving photographic plate. For torsing the wire the muscle is attached to a lever arm 10 mm. long fixed at right angles to the wire's axis at a given distance from the clamp holding the wire. The lever arm has points for attachment of the muscle at one-third, two-thirds, and full length of the arm. The excursion of the focussed image on the photographic plate amplified the excursion of the lever arm at these three points 560, 310 and 205 times respectively. Excursion of the image completely across the recording plate involved in all the three cases a torque of the wire of less than 3°. Within the range of torsions employed the excursion of the images for equal increments of pull (tension) was sensibly equal. The actual values of the tension scale were suitably adjusted by employing steel wire of different diameter without alteration of length. The range of tension scale employed varied from 1,000 grms. per 18 mm. excursion of the image to 100 grms. per 24 mm. excursion.

The natural vibration period of the myograph was in no case less than 1600 per sec. The damping was high; under sudden release (by breaking of the


St.
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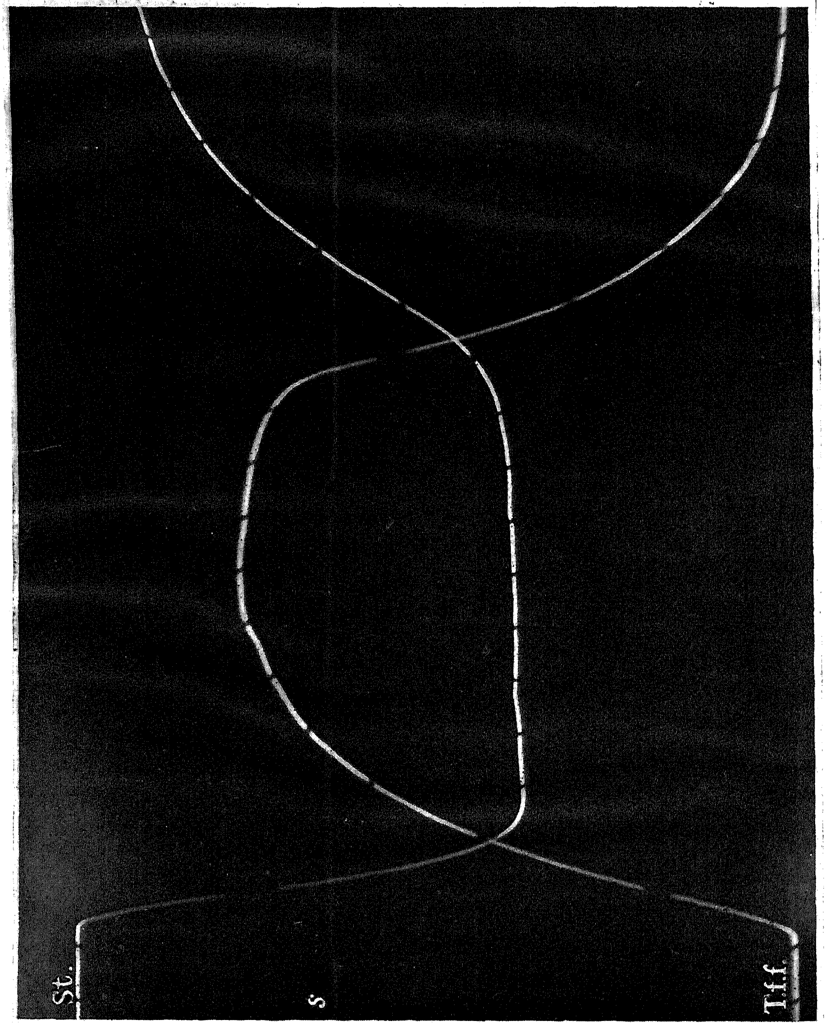
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muscle-tendon) from a tension of 5·5 kilos. and an excursion of 910 mm. magnifying 560, the image was steady again at zero in rather less than 0·015 sec.

With this arrangement a tetanic contraction developing as much as 6·5 kilos. and yielding a record of more than 110 mm. excursion on the plate permitted movement of the muscle-tendon by only 0·2 mm., *i.e.*, less than 0·2 per cent. of the length of the hip flexor employed or knee flexor employed, and less than 0·1 per cent. of the length of ankle flexor employed.

I am indebted to Mr. J. O'Neill of this Laboratory for his care and ingenuity in execution of the myograph. Its mirror device is similar in principle to that of Keith Lucas (3, *cf.* also 1, 2) for registration of isotonic contractions in excised frog muscle.

For recording time a Rayleigh magnetic wheel was used, revolving between the light source and illuminated slit, thus breaking the myographic image 50 times a second. Signal records were given by attaching to the armature of an electromagnet key which shorted the stimulating circuit, a mirror illuminated from a separate light source but focussing its image upon the recording plate.

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(3) Keith Lucas, 'Jnl. of Physiol.,' vol. 21, p. 353 (1904).

(4) Langley, 'Jnl. of Physiol.,' vol. 12, p. 347 (1891).

(5) Sherrington, 'Jnl. of Physiol.,' vol. 40, p. 28 (1910).

(6) Sherrington, These 'Proceedings,' B, vol. 92, p. 245 (1921).

(7) Sherrington, 'Integrat. Action of the Nerv. System,' London and New York, p. 127 (1906).

DESCRIPTION OF PLATE 8.

Concurrent contractions (isometric) of hip-flexor, *tensor fasc. femoris* (*T.f.f.*) and knee-flexor, *semitendinosus* (*St.*), in spinal flexion reflex (cat), evoked from *nerv. cutan. externus.*

FIG. 1.—*Tensor f. fem.* develops 2210 gm. tension, *semitend.* develops 710 gm.; tendon movement magnified by 310 for *tens. f. f.*; 560 for *semitend.* Stimulus, double shocks 90 a sec. at 14 cm. coil distance; stimulus period signalled (*s.*) direct by shorting key on plate. Time marked in 0·02 sec. by breaks in myogram line. Initial tension for both muscles approximately 40 gm. Time-relations of development of the plateau and of after-discharge are seen to differ between the two muscles.

FIG. 2.—Similar to above, but stimulus at 15 cm. coil. Tendon movement magnified by 205 for *tens. f. fem.* with tension of 200 grm. per cm. ordinate, and by 310 for *semitend.* with tension of 62 gm. per cm. ordinate. Initial tension about 30 gm. for *tens. f. f.*, about 20 gm. for *semitend.* The reflex latency of the mechanical response is for both muscles nearly the same, but that of *semitend.* is the longer.

A Further Note upon "Inter-traction."

By Sir Almroth E. Wright, M.D., F.R.S.

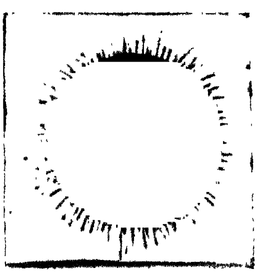
(Received, June 22, 1926.)

(Plate 9.)

I pointed out in a previous communication (vol. 92 (1921)) that a mixture of fluids can be brought about, not only by the operations of mechanical convection and diffusion, but also by the impulsion of a force, which can very rapidly carry down a lighter overlying fluid into a heavier underlying fluid in the form of characteristic pseudopodial streamers, conveying at the same time the heavier underlying fluid into the lighter superjacent fluid in the form of a palisade of ascending streams. I have ventured to call the agency, by which this reciprocal in-streaming is produced, *inter-traction.*

These phenomena, which I described as occurring when salt, and also sugar, solutions are brought into contact with albuminous solutions, can, as Schoneboom ('Proc.,' A, vol. 101 (1922)) showed, be obtained also with a wide range of substances; they have been ascribed by him to the operations of negative interfacial tension, and identified with phenomena theoretically anticipated by Clerk-Maxwell. Adam and Jessop, in a further communication (B, vol. 98 (1925)), have insisted that the pseudopodial streaming is attributable to operations of diffusion and resulting changes in specific gravity, and they have stressed the point that the characteristic appearances can be obtained only when the lighter is superposed upon the heavier fluid, and not when the fluids are disposed side by side. In view of the fact that the conclusion that horizontal streaming cannot be obtained rests only upon experiments conducted by filling fluids of different specific gravity into adjoining cell compartments and then removing the dividing wall, it seemed desirable to try for horizontal inter-traction with a technique which would get rid of the complication of heavier fluid sinking to the bottom and lighter going to the top of the vessel, and would allow of accurate and deliberate observation. The quite simple technique now to be described satisfies these desiderata.

A disc of filter paper is fixed, with a minute pellet of plasticine, to the surface of a cover-glass of larger diameter, and the four edges of the cover-glass rimmed with paraffin, in such a manner as to permit the cover-glass with attached disc being conveniently floated upon any desired fluid. The filter paper is impregnated with an albuminous fluid, such as serum, coloured with an anilin dye. The cover-glass is now set afloat upon a watch-glass of hypertonic salt solution (conveniently 4·5 per cent. solution of NaCl, as being a little heavier than serum). The streaming effects characteristic of inter-traction come into view almost immediately, presenting at first the appearance shown in photos 1 and 2. The streamers then spread outwards on the surface, until after about 5 minutes they extend far beyond the limits of the cover-glass (figs. 3 and 4).



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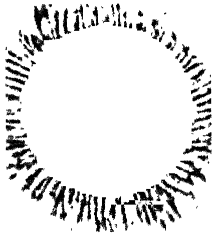

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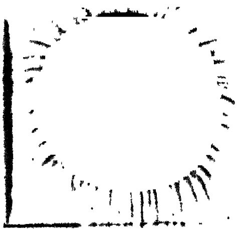

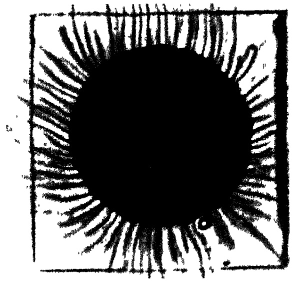

(Facing p. 268.)

Observations on the Resolution of Stasis in the Finer Blood Vessels.

By Howard Florey (John Lucas Walker Student).

(Communicated by Sir Charles Sherrington, O.M., F.R.S.—Received May 21, 1926.)

(From the Department of Anatomy, Cornell Medical School, New York.)

The sequence of events resulting in the production of stasis has been frequently described, but the processes involved in recovery from this state are more difficult to observe, and it is only by chance that such a recovery can be witnessed. The following observations have been made on the capillary circulation in the mesentery of the white rat.

A trough made from sheet tin was fashioned to accommodate the body of the rat. In one side of the trough, at the appropriate place, a rectangular piece of metal was cut away so that the gut with its mesentery attached could be withdrawn through it. To the large trough a tray of the same material was soldered. The tray was furnished with a circular hole about 1·5 cms. in diameter, in the bottom, the hole being covered with a glass slip cemented to the metal by means of de Khotinsky's cement. A moist chamber consisting of two parallel strips of glass was placed above this opening. To the top of these strips was cemented a small glass tube bent into the shape of a horseshoe. (See figs. 1-3.)

The whole apparatus was then mounted on a mechanical stage of a microscope by which means it could be moved accurately. The circular hole in the tray was so arranged as to be over the lens of the condenser. This sub-stage condenser had an especially long focal distance for use with hanging drop

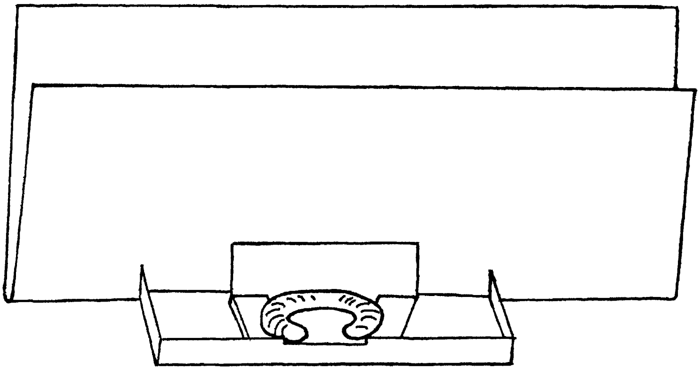

Fig. 1 —Trough and tray.

preparations. The type of microscope used had a large curve in the supporting arm so that the trough containing the rat fitted in snugly between the objectives and the microscope arm. The rat was anæsthetised with urethane, subcutaneously injected, and an incision made through the right flank through which the gut and its attached mesentery could be withdrawn. The animal was then placed in the trough and the incision arranged opposite the rectangular hole in the side of the trough. The gut was withdrawn with padded forceps and the mesentery spread over the glass ring. Some drops of warm saline were then placed on top of the spread mesentery and a round cover slip applied. The surface-tension at the interface between the mesentery and the glass caused the former to be flattened out on the under side of the cover slip. The tray was filled with saline, which also ran into the moist chamber, so that the mesentery was kept moist from above and below. The whole apparatus was placed in a warm box during the period of observation.

This apparatus was constructed so that the capillary vessels could be manipulated from below by means of a Chambers' micro-dissection apparatus. (See figs. 2 and 3.)

The small vessels of the rat's mesentery are very easily sent into stasis

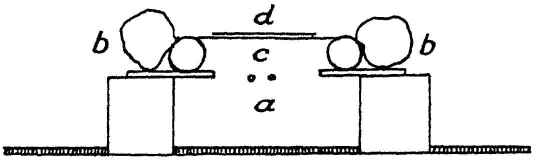

FIG. 2.—Tray, Elevation: *a*, Micro-dissection needle and pipette; *b*, intestine; *c*, mesentery; *d*, cover slip.

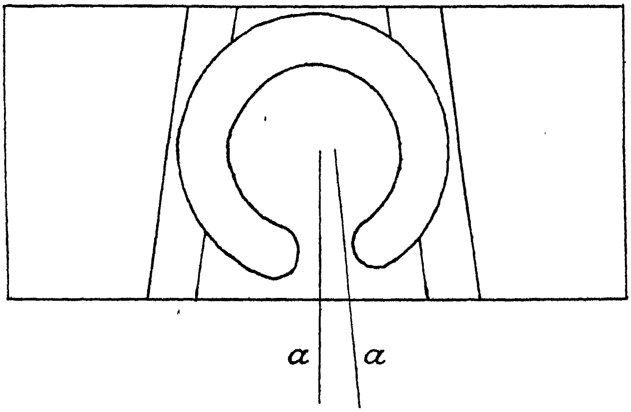

FIG. 3.—Tray, Plan *a*, Micro-dissection needle and pipette.

by rough handling or partial drying. One is, however, not fortunate enough to observe recovery from this often, as the degree of the initial damage cannot be regulated, and hence the time required for recovery.

In observing this mammalian preparation one is impressed by the greater rapidity, in fact, the more dynamic appearance, of the circulation compared to that of a frog, the animal most frequently used for such observations. I think that it is on account of the much greater activity of the finer circulation that it is possible to observe the recovery from stasis in a convenient period of observation. That such a recovery does occur is evident, when it is realised that after any abdominal operation the fine circulation in the surface of the viscera handled is in a state of stasis. But recovery occurs from this completely.

It seems to be established that during the production of stasis the capillary walls become more permeable, thus allowing the contents to become more concentrated; in fact all the fluid may be drained away, leaving a transparent mass of corpuscles. Before recovery can occur it is evident that the endothelium must regain its relative impermeability. It would seem unlikely that the changes occurring in this process could be observed microscopically.

For a description of the sequence of events it is convenient to consider an area in which some of the blood vessels have recovered their flow.

In the vessel A (fig. 4) is a rapid stream in which the individual corpuscles cannot be distinguished, nor any pulsatile flow observed. The capillary B is in a condition of complete stasis, the heavily shaded portion being transparent. The space x contains plasma with a few discrete corpuscles floating in it. In

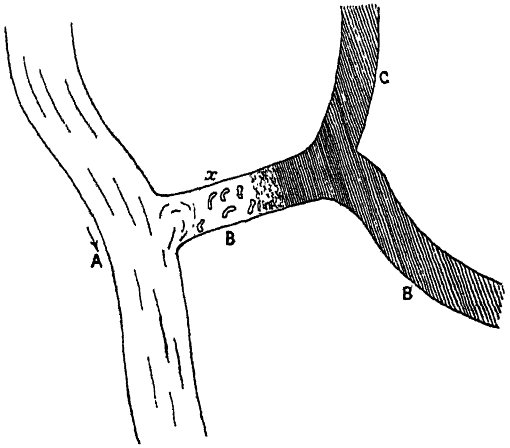

FIG. 4.

this portion of plasma is visible a very marked to and fro oscillation synchronous with the heart beat. At the junction of A and B is a small eddy, in which red and white corpuscles and some coccus-like bodies (blood platelets ?) revolve. These are whirled round and round to escape into the main stream, being then replaced by others.

At each pulsation of the plasma in the vessel B some of it is forced between the compacted red cells at the end of the column in stasis, so that there appears a zone which has ceased to be transparent and in which the outlines of the constituent cells can be seen. The red cells one or two at a time are gradually loosened from the end of this partially broken up column. These float down the plasma leading to the freely flowing vessel, where they become caught in the eddy and are whisked away. This process of attrition at the densely packed homogeneous-looking mass of corpuscles results in the canalising and eventual re-establishment of the flow in that vessel. The last clearing occurs with a series of jerks whereby the last portions of the compacted corpuscles are quickly pushed out. The process is then continued in the vessel C, and so on, till the circulation is completely re-established all over the area.

It is noteworthy that there are no white cells adhering to the walls after the circulation has recommenced, but these gradually appear after the lapse of 30 to 60 minutes.

Another process observed is as follows: The capillary B (fig. 5) contains a mass of cells which has been partially repermeated with plasma, so that the mass is not completely transparent. From time to time a mass of corpuscles is forced over the lip of the junction between A and B. The corpuscles adhere together till a sufficiently large mass projects into the lumen of A, when it is broken off and carried away in the stream. The first described method is, however, that most frequently observed.

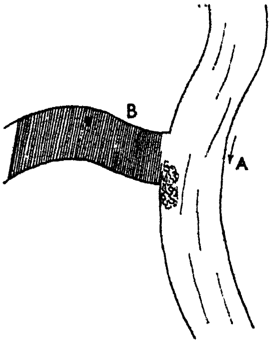

FIG. 5.

It is of interest to note that the corpuscles after they are freed from stasis do not present their usual disc shape, but some appear to be shaped like a concavo-convex lens. It would seem that their elasticity had been impaired, at least, temporarily. These observations would indicate that there is a considerable capillary pulse, which, though not visible in a freely flowing vessel, becomes apparent in a side vessel which is blocked.

There was no appreciable diminution in the calibre of the vessels after the mass of corpuscles had become canalised and the vessels were carrying a free flow. That is to say, that the diameter of a vessel in stasis and one recovered therefrom may be the same, although the permeability of the wall is very different. It is difficult to reconcile with this observation Krogh's idea that the permeability of a capillary becomes greater when it is dilated, for in this case one has a widely dilated capillary which has been very permeable, and at a later time the same capillary with the same diameter becoming much less permeable. It would seem just to conclude that there is some other factor at work changing the state of permeability. This observation gives support to those made by Lewis* on the reactions of skin capillaries, from which he arrived at the same conclusion.

I am indebted to Dr. Chambers and Dr. Reznikoff for much assistance and advice in the preparation of the apparatus.

Experimental Studies on the Differentiation of Embryonic Tissues Growing in vivo *and* in vitro.—II. *The Development of the Isolated Early Embryonic Eye of the Fowl when cultivated* in vitro.

By T. S. P. Strangeways, Lecturer in Special Pathology, University of Cambridge, and Honor B. Fell, Ph.D., Beit Memorial Research Fellow.

(Communicated by Sir William Hardy, F.R.S.—Received May 26, 1926.)

(From the Laboratories of the Research Hospital, Cambridge.)

(Plates 10 and 11.)

Introduction.

In a previous communication (Strangeways and Fell, 1926) it was shown that if the undifferentiated limb-bud of the embryonic Fowl was cultivated *in vitro*, it underwent a considerable amount of progressive development. This capacity for independent development *in vitro* possessed by an isolated organ has been further investigated, and for these later experiments the writers have employed the early embryonic eye, a structure endowed with more complex potentialities than the limb-bud. As a result of these experiments it was found that the eyes of young Fowl embryos possess, in a remarkable degree, the faculty for self-differentiation *in vitro* and for "organotypic" growth as defined by Maximow (1925). The previous work on organotypic growth *in vitro* has already been briefly outlined in the writers' earlier paper and need not be discussed here.

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* Lewis, T., "Vascular Reactions of the Skin to Injury. Part 1.—Reaction to Stroking: Urticaria Factitia." 'Heart,' vol. 11, p. 119.

The expenses connected with the experiments described in this communication were met by the Medical Research Council, to whom the writers desire to express their thanks.

Technique.

The embryos used in these experiments were from pure-bred White Leghorn stock and had been incubated for 64–72 hours when removed from the shell.

Each embryo was taken aseptically from the egg. One eye was dissected out with cataract knives with the aid of a binocular dissecting microscope and planted *in vitro,* the other eye being left *in situ.* The head with the remaining eye was then severed from the body, fixed in Allen's solution and finally sectioned. In this way each culture was provided with a control.

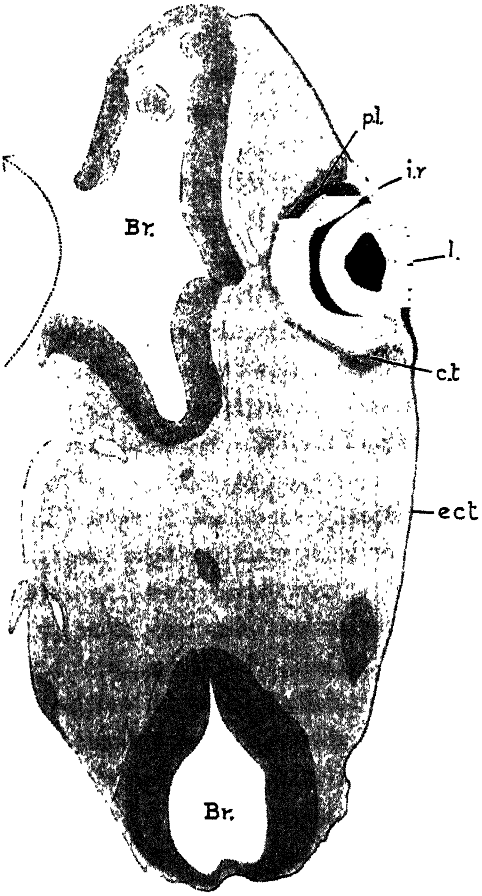
The cultures were grown in centrifuge tubes containing a mixture of ten drops of embryo extract and ten drops of plasma. The eyes were transferred to fresh medium at intervals of 48 hours. The technique employed is more fully described in the writers' previous paper (1926). In all, forty-seven eyes were explanted, but of these three were subsequently rejected owing to infection.

Explants were fixed at various periods of growth. Numerous fixing fluids were tried, but it was found that Flemming's solution (strong formula) without acetic acid and Allen's modification of Bouin's solution gave the best results. Complete serial sections of each explant were cut and the slides were stained with iron hæmatoxylin, Mallory's stain, iron hæmatoxylin and Van Giesen's stain, or safranin and picro-indigo-carmine.

Growth and Differentiation of Explants.

(1) *The Structure of the Eye at the Time of Explantation.*—The eye of embryos of 64–72 hours' incubation is a simple structure (text-fig. 1). The outer wall, or pigment layer, of the optic cup consists of a single stratum of cubical epithelium (fig. 1), which in some of the controls has not yet formed pigment, and in others contains sparsely scattered pigment rods. The inner wall of the cup, or retina proper, is formed of several layers of epithelial cells of which the outermost or germinal layer is in active mitosis. The choroid fissure is still open. The lens vesicle is either almost or completely closed, but in most cases

it is still attached to the ectoderm, so that a small area of the latter is usually removed along with the eye. In the less highly developed controls (text-fig. 1) the posterior wall of the lens vesicle is of the same thickness as the anterior wall, but in others the posterior walls have already begun to elongate to form the fibres of the lens. The optic cup is surrounded by undifferentiated mesenchyme, a layer of which usually remains adherent to the eye after it has been removed for explantation.



TEXT-FIG. 1.—Section through the head of an embryo (No 19) of 66 hours' incubation. One eye, the original site of which is indicated in the drawing by the dotted arrow, was removed and cultivated *in vitro* for 8 days ; it is shown in fig. 7 (Plate 11). The other eye was left to serve as a control and is seen in the section. Note the simple character of the eye with its vesicular lens (*l*) and undifferentiated retinal layers (*p.l.*, pigment layer ; *i.r.*, retina proper). The brain (*Br.*) is also shown.

(2) *Observations on the Living Explants.*—When first explanted the eyes are

minute, colourless bodies which are difficult to distinguish in the culture tubes. After about 20 hours' growth the optic cup has enlarged and is usually seen as a small, pale grey vesicle surrounded by a diffuse growth of mesenchyme. By the second day of cultivation the explant has about doubled its original size and the pigmentation is considerably greater, so that the eye is dark grey in colour.

When the eye is transferred to fresh medium, the greater part of the halo of mesenchyme is left behind with the old plasma, so that after the second or third change the optic cup is usually completely denuded of its mesodermal covering.

Until about the third day of cultivation the explant in most cases appears as a more or less spherical body completely enveloped by the dark grey pigment layer. After this period the pigment layer is almost invariably ruptured at one or more points by the rapid growth of the inner layer. The contour of the eye then becomes more and more irregular, as the inner layer, which has burst through the pigment epithelium, grows very fast and is thrown into irregular folds, whilst the pigment layer shows little increase in area and eventually appears as a mere knob on one side of the white colourless mass formed by the retina proper.

The eye cultures are usually somewhat elongated in an antero-posterior direction, the anterior region being marked by the dense black knob of pigment referred to above. The rate of growth is greatest between the second and fourth days of cultivation, after which it falls off rapidly. The maximum size is reached about the eighth day of cultivation, when the greatest length of the cultures is usually 1–2 mm. The largest culture obtained, which was almost spherical in shape, attained a diameter of about 2·5 mm., but on section this specimen was found to be largely cystic, an extensive area of the retina being reduced to a thin membrane showing no differentiation.

(3) *The Histological Development of the Explant.*—Histological examination of the cultures shows that during growth *in vitro* tissue differentiation takes place in a remarkably normal way and in the later stages frequently proceeds at almost the normal rate. As in the case of normal embryonic eyes (Weysse and Burgess, 1906) the degree of development reached by different eye cultures of the same age varies, and in the following account the writers have therefore described the typical stage of differentiation attained by the explants at given periods of cultivation.

Of the 44 cultures examined by histological methods all show pigment formation, and only the youngest cultures and four older explants fail to show some degree of differentiation of the inner or visual layer of the retina.

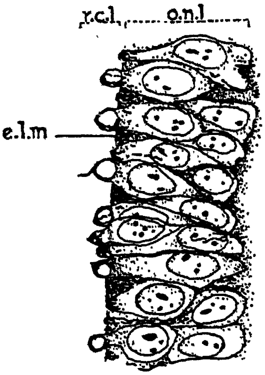
The youngest explants fixed had been growing *in vitro* for 19–21 hours. Cell proliferation in such specimens is extremely active, especially in the outermost or germinal stratum of the inner layer of the retina, where enormous numbers of mitotic figures are seen (fig. 2). Owing to this extremely rapid growth the internal layer of the retina or retina proper has become thrown into folds, the inner margins of which are usually in a more or less degenerate condition. In two of the three specimens fixed at this stage of cultivation the outer layer of the retina already contains large quantities of pigment rods, although in the controls pigment is either absent or present only as a few scattered rods. Mitosis is scarce in the pigment layer of the explants. The lens, although considerably larger, shows little change.

After two days' cultivation the lenticular zone, which gives rise to the iris and ciliary regions of the internal retinal layer, becomes distinguishable from the *pars optica*. In the *pars optica* the internal layer consists of several strata of undifferentiated cells, but in the lenticular zone it is composed of a single stratum of elongated, columnar cells. During subsequent development this columnar epithelium increases in extent, but undergoes no further structural change (fig. 3). All the tissues of the eye, including the developing fibres of the lens, contain numerous elongated mitochondria, which are extremely plentiful in the germinal layer of the retina (fig. 4).

In three-days old cultures the distinction between the optic and ciliary parts of the inner retinal layer is still more marked, as the former region is now about double the thickness of the latter. In the posterior portion of the retina, where development is usually further advanced than in the more anterior parts, the ganglion cells have sometimes begun to differentiate near the internal surface and are distinguishable from the adjacent cells by their larger nuclei and more plentiful cytoplasm. The remaining cells of the retina, which are densely packed together, consist of small oval nuclei enclosed by a mere film of cytoplasm. The pigment layer is now heavily pigmented. The posterior cells or fibres of the lens are considerably longer than those of the controls.

By the end of the fifth day of cultivation the ganglion cells are larger and more conspicuous (fig. 5), and are irregularly scattered in a loose network of fibres of which the innermost zone represents the nerve fibre layer and the peripheral zone the earliest rudiments of the inner plexiform layer. In some specimens the inner plexiform layer in the posterior parts of the eye is further advanced in development, appearing as a very narrow but compact band of closely woven fibres separating the ganglion cells from the cells of the inner nuclear layer. In the germinal layer, which might now be termed the outer nuclear layer, although not as yet sharply marked off from the inner nuclear layer, the majority of the mitochondria, originally elongated and diffuse, have now given rise in each cell to bunches of short stout rods similar to those described by Leplat (1913) in the normal visual cells of the embryonic Fowl. A small fat globule lies in the middle of each bunch.

After eight or nine days' growth *in vitro* all the zones of the retina are distinguishable (figs. 6 and 7). The internal limiting membrane and nerve fibre layer are well developed and the ganglion cells form an orderly row at the junction of the nerve fibre and inner plexiform layers. The inner plexiform layer is broad and compact and extends as far as the *pars ciliaris*. The inner cells of the inner nuclear layer have begun to differentiate into amacrine cells and are larger than the elements of the middle and peripheral regions, whilst the outer nuclear layer has become clearly defined from the inner nuclear zone. The principal feature of this phase of development is the appearance of the rods and cones (fig. 6) and text-fig. 2. The bases of the cells containing the bunch of short mitochondria and the fat globule referred to above penetrate the external limiting membrane as small round buds, similar to those described by Leplat (1913) in the case of the normal Fowl embryo; each bud is surmounted by a fine cytoplasmic process which represents the outer segment. In addition to the mitrochondrial masses present in the embryonic rods and cones, the cell bodies in the outer nuclear layer also contain mitochondria. In most specimens of eight or nine days' cultivation, the lens has become partially or completely degenerate, and in older cultures only the necrotic remains of the structure are to be found. In one eight-days' old explant, however (fig. 7), the lens is remarkably normal in appearance, has enormously increased in size (*cf.* text-figure 1, showing the control eye of this specimen, drawn to the same scale) and shows beautifully developed fibres.



Text-Fig. 2.

By the 13th day of cultivation the rods and cones have elongated considerably, the external plexiform layer is thicker and more distinct, and giant cells have appeared among the amacrine and ganglion cells.

In explants of 17 days' growth *in vitro* the rods and cones have begun to assume their adult form (fig. 8), being remarkably similar in appearance to the rods and cones of the day-old chick as figured by Leplat (1913). Various types of ganglion and amacrine cells, bipolar cells, horizontal cells and Muller's fibres with their spindle-shaped, rather darkly staining nuclei and brush-like internal terminations are all recognisable.

After the 17th day of cultivation the eyes undergo no further development and usually begin to degenerate. They may remain alive for another 15 days, but show increasingly large necrotic areas and a strong tendency to fat infiltration.

As previously stated (p. 276), the inner layer of the retina, owing to its more rapid growth, usually bursts through the pigment layer after the third or fourth day of cultivation. Sections show that this ruptured pigment layer in most cases folds back upon itself (fig. 3), the greater part of the visual layer of the retina being thus uncovered (fig. 7). As development proceeds, the pigment layer gives rise to complex folds and tubules, sometimes on an extensive scale (fig. 9), so that the actual growth of the pigment layer is more extensive than the examination of the living cultures would lead us to suppose. Some of the tubules so formed are blind, others have a lumen (fig. 10); in the less densely pigmented portions mitotic figures are sometimes common. This mode of growth of the pigment layer greatly distorts the development of the anterior portions of the eye, so that the iris can rarely be distinguished with any certainty.

In two cultures only are indications of an optic nerve distinguishable; in both cases this structure is somewhat indistinct. No trace of a pecten is found in any of the explants.

As described on p. 276, the spherical shape of the eye is lost by about the third day of growth *in vitro* and the optic cup becomes crumpled and irregular in contour. Sections show that the opposite walls of the cup are often closely approximated and occasionally fused (fig. 7), whilst complex infoldings, involving either the entire wall of the cup or the outer nuclear layer only, are also common. The lips of these folds usually become united in the older cultures, thus forming closed cavities lined by developing rods and cones.

The irregular cavities of the eye cultures contain a substance in which cells are rare and sometimes absent, and which, like white fibrous tissue, stains strongly with the anilin blue of Mallory's and the acid fuchsin of Van Giesen's stain. Possibly this material represents the vitreous body.

In those cases (p. 275) in which a small area of ectoderm has been removed along with the eye at the time of explantation, the ectoderm either rounds up into a small nodule (fig. 7) or remains as a sheet covering the lens and condensed pigment but not extending over the inner layer of the retina. In exceptional instances the entire eye becomes invested with ectoderm, which usually results in considerable degenerative changes taking place in the enclosed tissues.

The thin layer of mesenchyme which usually covers the optic cup when it is first explanted is for the most part lost, as described on p. 276, by the outwandering of the cells into the culture medium. A certain number of the mesenchyme cells, however, usually become incorporated in the mass of pigment epithelium and give rise to a loose stroma of connective tissue supporting the epithelial folds and tubules.

Discussion.

The capacity of the isolated embryonic eye for self-differentiation has already been studied by the method of grafts. Lewis (1907) has shown that the primary optic vesicle of Amphibian embryos grows and differentiates when grafted subcutaneously in abnormal situations. Hoadley (1924) found that the eye primordium of the embryonic Fowl grew and developed readily as a graft upon the chorio-allantoic membranes. The results obtained by the present writers have demonstrated even more clearly that the eye is a self-differentiating organ, since the histogenesis of the retina and lens may proceed almost normally *in vitro* where the optic cup is isolated from surrounding embryonic tissues and completely deprived of nerve connections and of a blood supply.

A comparison of the rates of growth and development of normal and explanted eyes shows that, as in the case of the limb-bud cultures, the differentiation of the organ *in vitro* advances much more rapidly than the growth. This disparity between rate of growth and rate of differentiation is more pronounced in the cultures of the eye than in those of the limb-bud, since in the case of the eye differentiation in its later stages may proceed almost, if not quite, as rapidly *in vitro* as in the eye socket of the normal embryo, whilst the growth rate becomes greatly subnormal after the first four days of cultivation. After 17 days' growth *in vitro*, therefore, the retina may exhibit a histological structure almost equivalent to that of the retina of a day-old chick, whilst the greatest length of the explant is only 1–2 mm. From this it appears that inhibition of normal cellular multiplication in an embryonic organ is not necessarily correlated with inhibition of normal tissue-differentiation in that organ.

Although the histological differentiation of the retina and, more rarely, of the lens may be almost perfect, the shape of the eye-ball is usually distorted beyond recognition. This is probably due in part to the absence of the firm supporting sclerotic, but the various abnormal mechanical influences brought into play by the conditions of the experiment are also, no doubt, largely responsible. Hoadley's statement with reference to the grafted eye, viz., that "the regulation of the parts to form a more or less perfect structure seems . . . to be dependent on various mechanical factors involved in the experiment," is probably equally applicable to the eye during cultivation *in vitro*.

Summary.

1. The eyes of Fowl embryos of 64–72 hours incubation were cultivated *in vitro* by means of a tube technique.

2. The explanted eyes showed remarkable powers of self-differentiation. Pigment, the different zones of the *pars optica* of the retina (rods and cones, inner and outer nuclear layers, inner and outer plexiform layers, ganglion cells and nerve fibre layer), the *pars ciliaris retinæ* and the fibres of the lens developed during cultivation *in vitro*.

3. Differentiation in its later stages proceeded *in vitro* at almost the normal rate, although the cultures obtained a maximum size of only 1–2 mm. From this it appeared that inhibition of normal cellular multiplication was not necessarily correlated with inhibition of normal tissue differentiation.

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DESCRIPTION OF PLATES.

All figures were drawn with the aid of a projecting prism. Figs. 1, 2, 3, 4, 5, 6 and 8 are drawn to the same scale.

a.c. ... = amacrine cells.
b.c. ... = bipolar cells.
c.t. ... = connective tissue.
ect. ... = ectoderm.
e.l.m. ... = external limiting membrane.
g.c. ... = ganglion cell.
i.l.m. ... = internal limiting membrane.
i.m.l. ... = inner plexiform or molecular layer.
i.n.l. ... = inner nuclear layer.
i.r. ... = inner layer of retina.
l. ... = lens.
M.f. ... = Muller's fibres.
n. ... = nucleus.
ne. ... = necrotic material.
n.f.l. ... = nerve fibre layer.
o.m.l. ... = outer plexiform or molecular layer.
o.n.l. ... = outer nuclear layer.
p.cil. ... = pars ciliaris retinæ.
p.l. ... = pigment layer.
p.op. ... = pars optica retinæ.
p.t. ... = pigment tubules.

Plate 10.

Fig. 1.—Section through the retina of the (control) eye of a 66-hours old embryo (No. 21). *Cf.* fig. 6, which is drawn from a section of the corresponding eye of the same embryo (No. 21) after it had been growing *in vitro* for 9 days. (Allen; iron hæmatoxylin.)

Fig. 2.—Section through the retina of a 19-hours old culture. The pigment has greatly increased in amount (only a few scattered rods occur in the control of this specimen). Mitosis is very active in the germinal layer. (Allen; iron hæm'n.)

Fig 3.—Section through the *pars ciliaris retinæ* of a 9-days old culture (No. 21). *Cf.* fig. 6, which shows the *pars optica* of the same specimen. Note the double layer of pigment epithelium formed by the folding back of the pigment layer after its rupture by the outgrowth of the retina proper. Numerous mitochondria are present in the cells. (F.W.A.; iron hæm'n.)

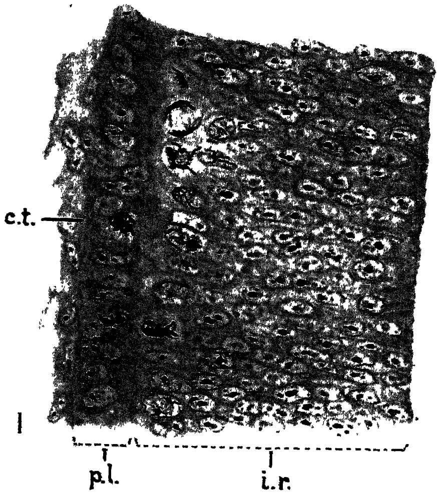
Fig. 4.—Section through the retina of a 2-days old culture, showing the aggregation of mitochondria in the germinal layer. (F.W.A.; iron hæm'n.)

Fig. 5.—Section through the retina of a 5-days old culture (pigment layer not seen). The ganglion cells and nerve fibres have begun to appear. Note the abundant mitochondria in the ganglion cells and in the cells immediately next to the external limiting membrane. (F.W.A.; iron hæm'n.)

Fig. 6.—Section through the retina of a 9-days old culture (No. 21). The pigment layer is not seen. All the zones of the retina with the exception of the outer plexiform layer are present. The buds of the embryonic rods and cones have penetrated the external limiting membrane. Each bud contains a mass of short, stout mitochondria. *Cf.* fig. 1, drawn from the corresponding (control) eye of the same embryo (No. 21) and note the great advance in differentiation. (F.W.A.; iron hæm'n.)

Plate 11.

Fig. 7.—Section of the eye (No. 19), which was removed from the embryonic head shown in the text-figure, after it had been cultivated *in vitro* for 8 days. This specimen is drawn to the same scale as the text-figure. Note the increase in size, the development of the lens, the demarcation between the *pars ciliaris* and *pars optica* of the retina, the appearance of the different zones of the retina, and the abundant pigment. The pigment



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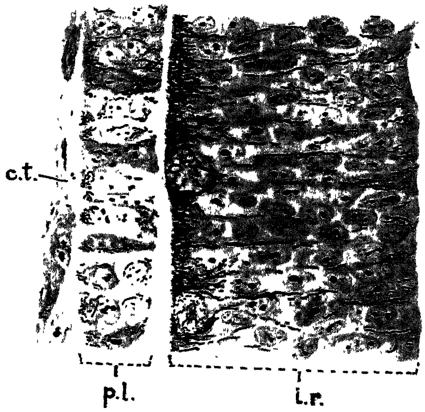

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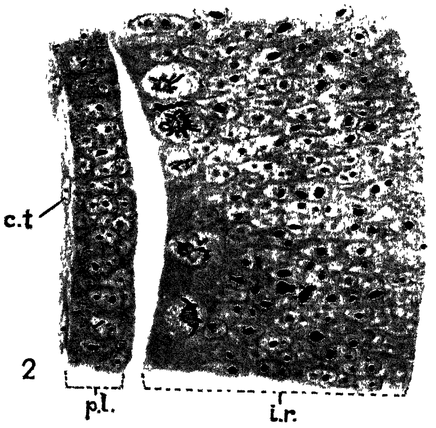

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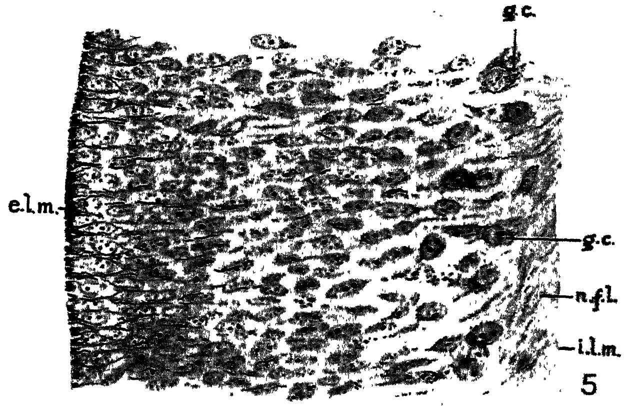

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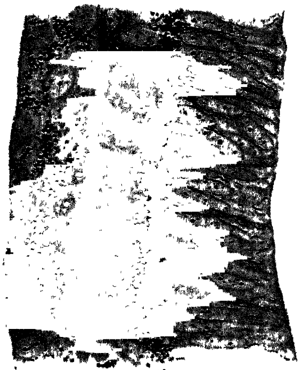

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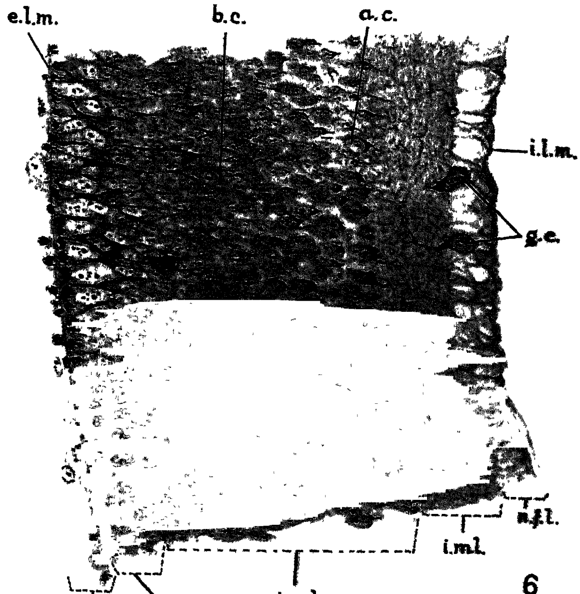

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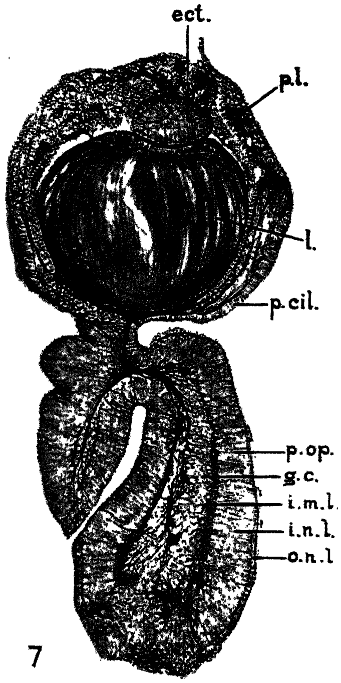

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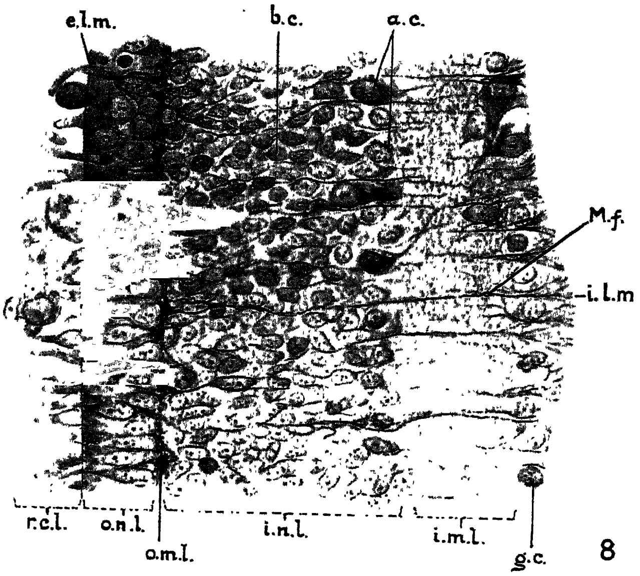

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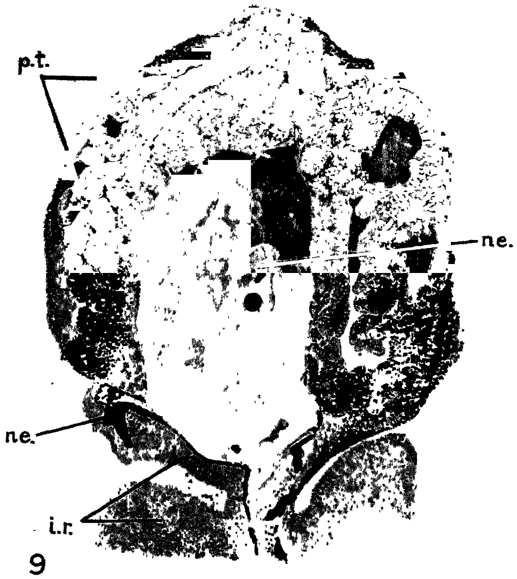

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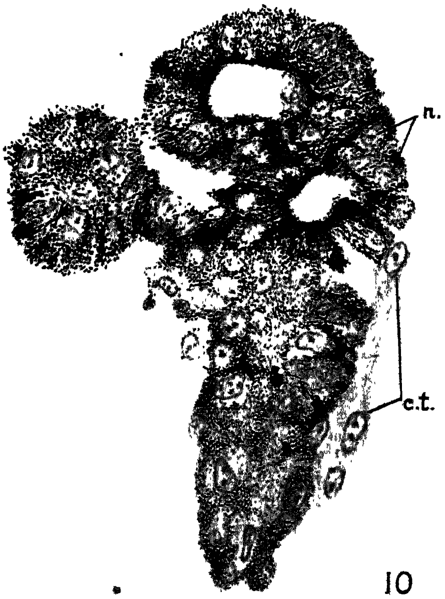

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H.B.F. *del.*

epithelium has become reflected upon itself, leaving the inner retina uncovered. (Allen; Mallory.)

FIG. 8.—Section through the retina of a 17-days old explant (pigment layer not seen). All the zones of the retina are well developed. This specimen shows the Muller's fibres and some of the nerve processes of the inner plexiform layer. (F.W.A.; Mallory.)

FIG. 9.—Section through the complex folds and tubules formed by the ruptured pigment layer of a 19-days' old culture. (Allen; iron hæm'n.)

FIG. 10.—Section of a pigment tubule from a 29-day sold culture as seen under high power. (Allen; hæm'n.)

The Effects of X-Rays upon Mitotic Cell Division in Tissue Cultures in Vitro.

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(Communicated by Sir William Hardy, F.R.S.—Received June 15, 1926.)

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Introduction.

The great majority of investigations on the effects of X-radiations on animal cells have been made under such conditions that the direct effects on the cell could not be separated from the indirect effects due to the reaction of the organism as a whole. The object of the experiments recorded in this communication was to investigate the direct action of X-radiation on cells when these reaction effects had been eliminated by using tissues grown *in vitro*. Special attention was directed (A) to investigating the effect of X-radiation upon cells in mitosis and upon cells about to divide; (B) to the question whether the effect of a given dose of X-radiation upon such cells is in any way dependent upon the quality of the rays.

In a previous communication (1) it was shown that when a tissue culture *in vitro* in which cell division is active is subjected to small doses of X-rays, a decrease in the number, and with somewhat larger doses a practically complete disappearance, of cells in normal mitosis was noted. These earlier experiments have been confirmed and extended. This inhibitory effect upon mitosis probably represents the most delicate and reliable test we have of biological response to irradiation, and for this reason has been adopted as the basis of the investigations here recorded.

The experiments described below were carried out partly at the Research Hospital, Cambridge, and partly at St. Bartholomew's Hospital, London. The writers wish to express their gratitude to the authorities of St. Bartholomew's Hospital for their kindness in permitting them to use their powerful deep therapy installation and for giving facilities for the work. We also wish to express our thanks to our assistant, Mr. V. C. Norfield, for his valuable help with the routine work in connection with these experiments.

The expenses connected with this research, both in Cambridge and London, were met by a grant from the Medical Research Council.

Technique.

The tissue used for cultivation was obtained from the choroid and sclerotic of chick embryos of 7 to 9 days' incubation. The tissue-fragments were grown in a hanging drop of mixed fowl plasma and embryo extract on a No. 1 cover-slip of soda glass which was sealed down with paraffin wax over a hollow ground soda glass slide.*

Cultures of the "second passage" which had been incubated from 18 to 22 hours were used for irradiation. Such cultures show a broad zone of cells among which can be seen a continuous succession of cells passing through the various phases of mitosis to form in each case two new daughter cells. In a single culture there are usually from 20 to 100 mitotic figures present at any one time. The division of a cell under these conditions occupies 40 to 50 minutes from the commencement of prophase to the complete formation of two full-sized cells (2). All cultures were examined microscopically on a warm stage before irradiation and only those which contained 20 or more cells in mitosis were used.

The cultures used for the first series of experiments were incubated in a Hearson's biological incubator at 38° C., but difficulties arose from the transference of the sealed cultures from warm air to the warm water of the bath in which they were irradiated (see below). In the later experiments therefore the cultures, immediately after implantation for the second passage, were placed in a hot-water bath of similar construction to that in which they were exposed to the X-rays, and were kept there at a constant temperature of 38° C. until required for irradiation.

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* The use of glass for mounting the cultures is not wholly desirable from a radiological point of view, and various other materials more suitable in this respect were tried in the hope that an efficient substitute might be discovered. Unfortunately it was found impossible to maintain a constant standard of growth when substances other than glass were employed.

During their exposure to the X-rays the cultures were incubated in water tanks made of tinplate. The dimensions of these tanks were 60 cm. long by 30 cm. broad and 20 cm. deep. Each was heated by an electric radiator placed under it, and its temperature controlled at 38° C. by a thermostat and motor-driven stirrer. The cultures were supported by an aluminium bridge which could be levelled, and were immersed in the water to a known depth of 10 to 15 mm. The bridge was so designed that four cultures could be similarly and simultaneously exposed, and so that the middle third of the slide on which the culture was growing was surrounded by water on all sides, including the lower surface.

The X-ray installations used both at Cambridge and London were induction coils with mercury-gas interrupters. For the exposures made with the lower voltage X-rays, a standard Coolidge tube with battery filament control was used, but for the higher voltages a deep therapy Coolidge tube with transformer filament control was substituted. The more powerful installation was a twin coil apparatus,* which could at will be used to give either the usual pulsating type of discharge, or (by the inclusion of thermionic rectifiers and condensers in the secondary circuit) a continuous high voltage discharge. Both types of discharge were used.

The exposures, or incident doses of X-rays given, were measured both at Cambridge and St. Bartholomew's Hospital by a Friedrich's iontoquantimeter, the two iontoquantimeters being calibrated against each other at various intervals of time. A description of the construction, calibration and use of this instrument is given by Kroenig and Friedrich (3). By the use of this instrument the " dose " or " exposure " received by an object placed in an X-ray beam is measured in absolute units. The unit employed, designated by "1 e," is that amount of energy of beam which will produce in 1 c.c. of air under normal conditions an amount of ionization that as a saturation current will cause a change of one electrostatic unit of quantity of electricity in the measuring system. The doses recorded throughout this paper are those which would be recorded by the iontoquantimeter if its ionization chamber had occupied the position of a culture during the period of irradiation. To distinguish these from measurements of the amount of X-ray energy *absorbed* by a specimen they have been called "incident doses."

After exposures of predetermined amounts the cultures were either fixed immediately, or incubated at 38° C. for 80 minutes and then fixed. The reasons for selecting 80 minutes for incubation have been discussed in a previous paper ((1), p. 377).

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* The neo-symmetric apparatus of Messrs. Reiniger, Gebbert and Schall, Erlangen.

The cultures were subjected to the following series of doses as measured in Friedrich's "e" units, 1e, 5e, 10e, 20e, 30e, 40e, 50e, 60e, 70e, 80e, 90e, 100e, 120e, 140e, and 160e. The complete doses were given in single exposures, the intensity being adjusted in each case to approximately 5e a minute. Usually four, but never less than two, cultures were exposed simultaneously. Although the standard of growth of the cultures employed was approximately constant, it is nevertheless a technical impossibility to obtain cultures in which precisely the same number of cells in mitosis are present at any given time. It was therefore necessary when determining the effect of irradiation by small doses to take averages of the number of mitotic cells in the controls as well as in the irradiated cultures. In the earlier part of the work a single culture was fixed as a control to each experiment, but in the later experiments the number of control specimens equalled that of the irradiated cultures. The reason for increasing the number of controls was the difficulty mentioned above of obtaining a large number of cultures containing approximately an equal number of cells in mitosis.

Results.

A series of experiments showed that if the cultures were stained immediately after irradiation no diminution in the number of cells in mitosis was found until, with the given intensity, a dose of 60e had been given.

Examination of cultures incubated for 80 minutes after irradiation, on the other hand, showed a definite decrease in the number of mitotic cells even after a dose of 1e had been given, and when a dose of 10e had been received, a very marked decrease in the number of mitotic cells was noted. Such specimens were found to contain an average of about 7 mitotic cells only as compared with over 50 in the controls, whilst an average of only 4–5 mitotic cells was observed in cultures which had received doses intermediate between 10 and 50e. After a dose of 50e a further fall in the number of mitotic cells was seen, the average numbers present being reduced to about 3 per culture. After doses of 60e and upwards mitosis was practically absent and an average of less than one cell in mitosis was found. It was further noted that after doses of 30e and upwards some of the mitotic cells present showed abnormalities such as fragmentation of the chromosomes, definite lag in the time of division or in the passage of the chromosomes to the poles of the spindle, and occasionally a breaking down cell was present. As the dose was increased, the proportion of these abnormal cells to those in which mitosis appeared normal, gradually increased, so that after doses of 60e practically every one of the few mitotic cells found was abnormal and breaking down cells were more numerous. With doses of between 60e and 150e, in addition to the rare abnormal mitotic cells, tripolar and multipolar cells were occasionally seen, and there was an increasing number of breaking down cells. It appears that vegetative cells pass through a phase immediately prior to visible prophase (1) during which the physiological processes of the cell are especially liable to be disturbed; if such a cell receives a dose of X-rays greater than 30e it will nevertheless enter mitosis, but the process of division will be of an abnormal type and may sometimes result in complete disruption of the cell.

Typical examples of the results obtained after 80 minutes' incubation are given in the Tables I, II, and III, in which are analysed a representative selection of typical experiments.

Table I.

Induction Coil apparatus giving a pulsating current in secondary circuit.

Coolidge Tube, Tungsten Target.

Focal distance = 15 cm. Area of field = full width of bath.

Secondary current = 3 milliamps. Filter = 1 cm. of water. Alternate spark gap — point and plane = 15 cm.

Exp. No.	E.312	E.312	E.311	E.309	E.309	E.310	E.310	E.299
Time for 10e	2 m. 5 s.	2 m. 5 s.	2 m.	2 m. 12 s.	2 m. 12 s.	2 m. 7 s.	2 m. 7 s.	1 m. 55 s.
Dose	1e	5e	10e	20e	30e	40e	50e	60e
	52 *30*	27 *30*	10 *36*	5 *102*	2 *102*	5 *49*	3 *49*	0 *50*
	57 *72*	26 *72*	5 *46*	7 *65*	4 *65*	4 *92*	2 *92*	0 *51*
	39 *57*	39 *57*	4 *63*	4 *83*	7 *83*	3 *95*	3 *95*	1 *54*
	29 *59*	26 *59*	9 *51*	7 *85*	4 *85*	4 *67*	4 *67*	0 *54*
	47 *44*	30 *44*	7 *46*	5 *68*	5 *68*	5 *60*	2 *60*	0 *51*
	34 *45*	38 *45*	5 *53*	4 *77*	5 *77*	3 *52*	4 *52*	0 *45*
	26 *60*	20 *60*	4 *61*	4 *78*	1 *78*	4 53	2 *53*	0 *50*
	52 *51*	43 *51*	7 *56*	2 *70*	5 *70*	5 *48*	2 *48*	1 *35*
Average	42 *52·2*	31·1 *52·2*	6·3 *51·5*	4·7 *78·5*	4·1 *78·5*	4·1 *64·5*	2·7 *64·5*	0·25 *48·5*
Average as percentage	80·3	59·5	12·2	6·0	5·2	6·4	6·6	0·5

Numbers in Roman figures represent number of cells in mitosis in irradiated cultures. Numbers in italics represent number of cells in mitosis in control cultures.

Table II.

Induction Coil apparatus giving a pulsating current in secondary circuit.

Coolidge Tube, Tungsten Target.

Focal distance = 30 cm. Area of field = full width of bath.

Secondary current = 4 milliamps. Filter = 0·5 mm. Zinc + 2 mm. asbestos + 2 mm, wood + 1 cm. water. Alternate spark gap — point and plane = 42 cm.

Exp. No.	E.317	E.306	E.306	E.301	E.304	E.304	E.306	E.303
Time for 10e	2 m. 23 s.	2 m. 9 s.	2 m. 9 s.	2 m. 8 s.	1 m. 59 s.	1 m. 59 s.	2 m. 9 s.	2 m.
Dose	1e	5e	10e	20e	30e	40e	50e	60e
	45 *75*	44 *56*	9 *56*	6 *22*	2 *63*	5 *63*	4 *56*	0 *80*
	37 *68*	38 *69*	5 *69*	6 *40*	8 *31*	5 *31*	3 *69*	0 *80*
	44 *41*	45 *60*	6 *60*	3 *24*	2 *71*	5 *71*	2 *60*	0 *84*
	37 *50*	30 *73*	10 *73*	3 *25*	4 *40*	5 *40*	3 *73*	1 *80*
	36 *48*	25 *63*	6 *63*	6 *66*	5 *37*	6 *37*	2 *63*	0 *79*
	32 *76*	37 *34*	8 *34*	3 *43*	1 *61*	2 *61*	2 *34*	2 *64*
	46 *70*	35 *35*	5 *35*	3 *45*	4 *40*	2 *40*	6 *35*	0 *63*
	50 *60*	47 *60*	9 *60*	5 —	1 *34*	2 *34*	2 *60*	2 *68*
Average	40·9 *61*	37·6 *56·2*	7·2 *56·2*	4·3 *38*	3·3 *47·1*	4 *47·1*	3 *56·2*	0·6 *74·7*
Average as percentage	67·0	66·9	12·8	11·3	7·0	8·5	5·3	0·8

Numbers in Roman figures represent number of cells in mitosis in irradiated cultures. Numbers in italics represent number or cells in mitosis in control cultures.

In order to investigate the cause of this gradual disappearance of mitosis, a series of cultures which had received doses of X-rays, varying from 10e to 60e, were kept under continuous observation, the microscope being enclosed in an incubator kept at 38° C. These observations on the living cultures showed that, even with doses of 60e, the cells which were already in some stage of mitosis before irradiation began, passed through the remaining phases and gave rise to two daughter cells. This was true even in the case of cells in very early prophase.

From these observations it appeared that the decrease in number and final non-appearance of cells in mitosis was not due to the injury, death or disintegration of cells whilst actually passing through division, but to the inhibition of those cells which normally would have entered mitosis during the period of irradiation. Thus, those cells which were already in mitosis at the beginning

Table III.

Continuous High Voltage Apparatus. Coolidge Tube, Tungsten Target. Focal distance = 30 cm. Area of field = full width of bath. Secondary current = 3 milliamps. Filter = 0·5 mm. Zinc + 2 mm. asbestos + 2 mm. wood + 1·5 cm. water. Alternate spark gap – point and plane = 38 cm.

Exp. No.	E.302	E.308	E.308	E.315	E.315	E.314	E.305	E.316
Time for 10e	1 m. 58 s.	2 m. 18 s.	2 m. 18 s.	2 m. 14 s.	2 m. 14 s.	1 m. 49 s.	2 m. 8½ s.	2 m. 8 s.
Dose	1e	5e	10e	20e	30e	40e	50e	60e
	60 *55*	42 *37*	7 *37*	4 *60*	5 *60*	4 *46*	1 *57*	1 *75*
	43 *70*	50 *75*	9 *75*	7 *45*	6 *45*	4 *54*	3 *50*	2 *68*
	48 *67*	17 *70*	11 *70*	6 *47*	4 *47*	2 *68*	4 *61*	1 *41*
	41 *70*	31 *20*	5 *20*	7 *51*	3 *51*	1 *54*	1 *35*	1 *50*
	40 *43*	33 *69*	3 *69*	9 *64*	3 *64*	6 *30*	1 *26*	0 *48*
	37 *55*	14 *74*	4 *74*	4 *80*	5 *80*	6 *36*	2 *58*	1 *76*
	64 *65*	30 *37*	8 *37*	5 *43*	3 *43*	2 *53*	3 *36*	1 *70*
	50 *57*	10 *37*	12 *37*	6 *57*	4 *57*	5 *38*	2 *43*	0 *60*
Average	47·8 *60·2*	*28·3* *52·3*	7·3 *52·3*	6 *56*	4·1 *56*	3·7 *47·4*	2·1 *45·7*	0·7 *61*
Average as percentage	79·4	53·1	13·7	10·6	7·3	7·8	4·6	1·1

Numbers in Roman figures represent number of cells in mitosis in irradiated cultures. Numbers in italics represent number of cells in mitosis in control cultures.

of irradiation and those which were on the point of entering mitosis completed division and formed two cells.[*] Owing, however, to the fact that the action of the X-rays prevented further cells from passing into mitosis, a fresh supply of normal mitotic cells to replace those which had completed division was not forthcoming, and therefore (as is easily understood) the number of normal mitotic figures rapidly decreased and ultimately disappeared. The apparently contradictory results found in the case of cultures fixed after irradiation followed by 80 minutes' incubation, as compared with those obtained when the cultures were fixed immediately after irradiation, could thus be explained.

Cultures stained immediately showed the usual number of cells in mitosis even after receiving 60*e*, because in these experiments the time required for giving this dose was considerably shorter than the time required for the cells to pass through the various phases of division and form two daughter cells.[†]

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[*] *Cf.* Strangeways and Oakley ((1), p. 379).

[†] *Cf.* Strangeways and Oakley ((1), p. 380).

The writers wish to emphasize this observation and to point out that any deductions concerning the effect of X-rays upon mitosis, which are based upon experiments in which the cells are fixed immediately after irradiation, may be fallacious.

It was important to determine whether the cells which had been prevented from dividing by the action of the X-rays were permanently affected, and accordingly experiments were made in which cultures were given various doses of between 10e and 60e and returned to the incubator for different periods. It was found that after 4–5 hours' incubation the cultures recovered; they then contained a number of cells in mitosis* and the tissue could be sub-cultured an indefinite number of times.

A glance at the Tables I, II and III will show that even so small a dose as 1e has an inhibitory effect on mitosis, and therefore no evidence of any stimulating effect after irradiation was obtained.

Effect of Variation in Quality of Radiation.

A series of experiments was carried out in order to test whether the effect of X-radiation on cells varies with the quality of the rays used. The cultures were exposed under water in the bath described above and the tension exciting the X-ray tube was varied from approximately 90 kilovolts to 220 kilovolts. The filtration was as follows (the other conditions being those stated in Tables I, II and III):—

Pulsating Discharge, Tungsten Target.

15 cm. spark gap with	1 cm. depth of water	only as filter.
30 cm. ,, ,,	1 cm. ,,	,, ,,
38 cm. ,, ,,	1 cm. ,,	and 0·08 mm. copper as filter.
40 cm. ,, ,,	1 cm. ,,	and 0·5 mm. zinc as filter.
42 cm. ,, ,,	1·5 cm. ,,	and 0·5 mm. zinc + 2 mm. wood + 2 mm. asbestos as filter.

Constant High Voltage,† Tungsten Target.

38 cm. spark gap with 1·5 cm. depth of water and 0·5 mm. zinc + 2 mm. wood + 2 mm. asbestos as filter. The incident doses of X-rays were measured as before by a Friedrich's iontoquantimeter.

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* *Cf.* Strangeways and Oakley ((1), p. 380).

† In this case peak voltage as measured by spectrograph equals 171 kilovolts.

It was found that within these limits of voltage and filtration, and for doses given with the intensities mentioned and measured in the above manner, variation in quality of the incident rays had no appreciable influence upon the dose required to prevent the onset of mitosis. Comparison of Table I (in which series of experiments spark gaps of 15 cm. were used) with Tables II and III (in which spark gaps of approximately 40 cm. were used) illustrates this statement. It must be clearly understood that this deduction does not exclude the possibility of some wave-lengths being more effective than others when *absorbed* by the cell. We wish to emphasize that the measurements given are, so far as the cultures are concerned, only measurements of the energy of the *Incident Beam* of X-rays, and that at this stage no data can be given concerning the amount of energy which must be *absorbed* by different parts of a cell, or by the cell as a whole, to produce any biological effect.

The question now arises as to whether these results can justifiably be interpreted as indicating that the observed effects exhibit a true independence of the quality of the incident radiation. For this to be the case it is necessary either to adopt the Friedrich iontoquantimeter as a standard dosimeter, or to show that its indications agree with those of an accepted standard instrument.

Physicists are agreed that the best way of measuring the intensity of a beam of X-rays is to measure the ionization it produces in air, when any additional ionization, due to the action of the beam on the walls or electrodes of the ionization chamber used, is eliminated. No general agreement, however, has yet been arrived at as to the dimensions or specification of a standard ionization chamber to be used for this purpose.

In the present case, through the kind co-operation of Prof. E. A. Owen, the Friedrich iontoquantimeter was calibrated against a "standard" ionization chamber made of tinned iron, which was 1 metre in length, 10 by 10 cm. in cross-section, and open to the atmosphere at each end.

The active electrode of aluminium was situated at a distance of approximately 20 cm. from the open end nearer the X-ray tube and was 2 cm. long in the direction of the beam and 5 cm. wide. This electrode was furnished with a "guard ring" extending the whole length of the chamber. The wall of the chamber constituted the other ("earthed") electrode; the width of the air-gap was 8 cm. The beam of X-rays was limited to a fine pencil by means of two apertures (diameters 3 and 1·5 mm., respectively) in two lead screens 14 cm. apart, the screen nearer to the ionization chamber being 10 cm. distant from its proximal open end.

The iontoquantimeter ionization chamber was clamped to a wooden stand

and placed in an X-ray beam, whose cross-section was limited by diaphragms so as to exceed slightly the exposed surface of the chamber.

A summary of the conditions and the results of this calibration is as follows:—

American Deep-Therapy Coolidge Tube with Tungsten Target.

Induction Coil with Mercury-gas interrupter and Kenotron rectifier delivering pulsating current to the X-ray tube.

Peak voltage measured by alternate gap between two spheres of 10-cm. diameter.

Peak Voltage.	Filter.	Ionization in Standard Chamber in scale divs. of electromr. when Iontoquantim. records 1e.
210	0·5 mm. Zinc 2 mm. Asbestos 2 mm. Wood	6·6
190	do. do.	5·1
160	0·08 mm. Copper 1 cm. Paraffin wax	5·9
90	1 cm. Paraffin wax	4·9

Although great care was exercised in making these measurements, the difficulties attending accurate work of this nature are so manifold that the highest precision is not claimed for them. They tend, however, to confirm the measurements made by Behnken (4) on a similar instrument, and serve to show that the variation in the dose required to produce a given biological response to radiation over the range of these experiments is less than 40 per cent.

Conclusions.

1. The earliest recognizable effect of X-radiation upon growing cells is the temporary inhibition of the onset of mitotic division in the majority of those fully formed vegetative cells which are about to divide.

2. Cells actually undergoing mitosis are unaffected by X-radiation.

3. It appears that vegetative cells pass through a phase immediately prior to visible prophase during which the physiological processes of the cell are especially liable to be disturbed; if such a cell receives a dose of X-rays greater than 30e it will nevertheless enter mitosis, but the process of division will be of an abnormal type and may result in complete disruption of the cell.

4. Variation in the quality of the incident beam of X-rays, within the limits of the doses and times here recorded, appears to have no appreciable effect on the results when the dose is measured with a small horn ionization chamber. If, however, the doses are measured by a " standard " air chamber, a possible increase in efficiency of less than 40 per cent. in favour of the longer wave-lengths is suggested.

5. No evidence was obtained of any stimulating effect of X-rays on cell division.

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(2) Strangeways, T. S. P., 'Roy. Soc. Proc.,' B, vol. 94, p. 137 (1922).

(3) Kroenig and Friedrich, 'Die Physikalischen und Biologischen Grundlagen der Strahlentherapie.'

(4) Behnken, 'Zeits. für Technische Physik,' Nr. 1 (1924).

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*The Antiseptic Properties of the Amino Derivatives of Styryl and Anil Quinoline.**

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Biological Section.

Previous communications have dealt with the antiseptic properties of compounds of the pyridine, quinoline, acridine and phenazine series,† and also of the apocyanine, carbocyanine, and isocyanine groups,‡ the object being to investigate relationships between chemical constitution and antiseptic properties. The following work comprises the preparation and examination of a large number of styryl and anil quinoline compounds, considerable variations in chemical structure being involved. The authors have previously drawn attention* to the possible influence of a particular chemical grouping consisting of a chain of alternate double linkages joining two nitrogen atoms, one being basic and trivalent, and the other the pentavalent nitrogen of a quaternary ammonium group. This arrangement is present in acriflavine (2·7 diamino acridine methochloride), and also in the cyanines, in all of which the antiseptic action is not diminished by the presence of serum.

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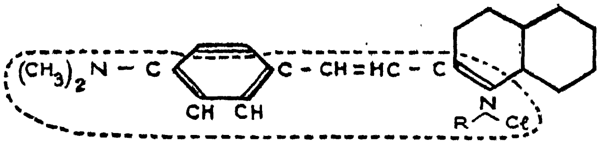
* The work reported in this communication was done with the support of the Medical Research Council.

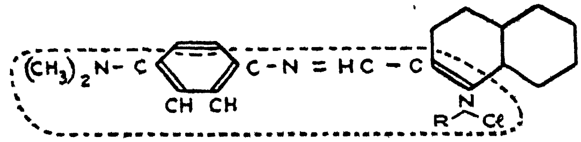
† Browning, Cohen, Gaunt and Gulbransen, 'Roy. Soc. Proc.,' B, vol. 93, p. 329 (1922).

‡ Browning, Cohen, Ellingworth and Gulbransen, 'Roy. Soc. Proc.,' B, vol. 96, p. 317 (1924).

As shown by the graphic formulæ, the styryl compounds also include a similar system, whereas in the anils an additional nitrogen atom is interposed in the connecting chain in place of a —CH= group. This system will be subsequently described as the "alternate linkage system."



Dimethylamino styryl compound.



Dimethylamino anil compound.

Antiseptic power was estimated by the same methods as those used in the previous investigations,*†‡ since with compounds of the present series also the lethal action on bacteria is exerted somewhat slowly and is preceded by bacteriostatic effects. The results are recorded numerically, the highest concentration which permits vigorous growth, and the lowest producing sterility, as tested by subculture of a loopful of the mixture on agar, being given. In addition, a record has been kept of the lowest concentration which suffices to inhibit growth, *i.e.*, to prevent the development in the medium of any definite turbidity obvious to the naked eye. Special attention has been paid to this "inhibitory concentration," since often it appears to yield a more satisfactory index of the antiseptic property of a slowly acting bactericidal substance than does the sterilising concentration. Thus, although the sterilising concentration may vary considerably in repeated tests of the same substance, the concentration at which inhibition begins tends to be more constant. A striking example of this is shown in Table I.

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* Browning, Cohen, Gaunt and Gulbransen, 'Roy. Soc. Proc.,' B, vol. 93, p. 329 (1922).

† Browning, Cohen, Ellingworth and Gulbransen, 'Roy. Soc. Proc.,' B, vol. 96, p. 317 (1924).

‡ Browning, Cohen, Ellingworth and Gulbransen, 'Brit. Med. Journ.,' vol. 2, p. 326 (1923).

Table I.—Antiseptic action of 2 (*p*-dimethylamino-styryl)-6-butyryl-amino quinoline methochloride for *Staphylococcus aureus*. Result read after the mixtures had stood at 37° C. for 48 hours.

Medium: Peptone water.

	+	±	—
Experiment *a*....	1 : 1000000	1 : 400000	1 : 200000
Experiment *b*....	1 : 1000000	1 : 400000	1 : 10000

In Experiment *b*, of course, all concentrations from 1 : 400000 to 1 : 20000 caused inhibition of growth.

In the tables the following designations are used :—

+ = free growth of the organisms with the development of marked turbidity of the medium.

± = inhibition of growth, the medium being only faintly turbid or remaining unclouded, but in the latter case sub-cultures yielding growth.

— = complete sterility, as tested by the absence of growth in subcultures.

General Considerations.

There is a close parallelism in the properties of the fundamental compounds 2 (*p*-amino-styryl) quinoline methochloride (1) and 2 (*p*-amino anil) quinoline methochloride (41), both being moderately powerful antiseptics for *Staphylococcus aureus,* but weaker in their action on *B. coli*. The changes in chemical constitution which have been studied produce in general closely similar effects upon the antiseptic properties of the two series, the most outstanding difference being the very strong action on *B. coli* which is developed in certain members of the anil group, *e.g.*, 52, 59, 69, 68. This distinction is illustrated, in a more or less marked degree, by the following pairs of anil and styryl compounds :—47, 14 ; 48, 16 ; 58, 22 ; 59, 24 ; 65, 27 ; 66, 28 ; 67, 29 ; 68, 30 ; 69, 31.

The two lepidine derivatives (82 and 40) form an exception to this rule, the styryl compound being the more active, but neither is powerfully antiseptic.

Certain of the compounds described, especially members of the styryl series, possess marked trypanocidal action in infected animals. Observations on this property will form the subject of another communication.

Substitution in the Benzene Nucleus.

For purposes of comparison, compounds unsubstituted in the benzene nucleus were prepared. The styryl compound* (83) is so sparingly soluble in water that only slight significance can be attached to the result, although the action on staphylococcus is considerable in watery medium ; but the potency in serum is less than that of the corresponding aminostyryl (4), and much below that of the dimethylamino compound (16). The analogous anil (91) is actively antiseptic for staphylococcus in watery medium, but has little effect in serum. It has very slight action on *B. coli* in either medium. 2 Anil 6 acetylamino-quinoline methochloride (92) is practically devoid of antiseptic action. On the other hand, 2 anil 6 dimethylamino quinoline methochloride (93), although not very potent in watery medium, has a powerful action against *B. coli* in serum.

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* A 0·5 per cent. solution of this substance was obtained in 20 per cent. alcohol acidulated with hydrochloric acid.

Primary amino groups.—The amino styryl and anil compounds, as already mentioned, have only a moderate antiseptic action on *Staphylococcus aureus* both in peptone water and in serum, the effect on *B. coli* being somewhat weaker. 2 (*p*-amino-styryl) 6-methyl quinoline methochloride (4) has a distinctly more powerful action than the corresponding ortho and meta compounds (2, 3). It may be noted that substitution in the ortho position decreases the length of the alternate linkage system, whereas meta substitution also interferes with its continuity. In view of the very moderate potency of all three isomers, however, the comparison does not afford very definite evidence of the effect of such changes.

Tertiary amino groups.—In both series of compounds, the substitution of a tertiary for a primary basic group in the benzene ring tends to increase the antiseptic action, although to a varying degree, as shown by comparison of the following pairs of substances :—

Styryl Series ..	14, 1 ; 16, 4 ; 21, 6 ; 24, 8 ; 27, 9 ; 37, 11.
Anil Series ..	47, 41 ; 48, 42 ; 56, 43 ; 59, 45 ; 75, 46.

This is especially the case with the anils and *B. coli.* 2 (*p*-Dimethylamino anil) quinoline methochloride (47) is at least five to ten times more potent than the primary amino compound (41). Diethylamino anils have a very similar action to the dimethylamino derivatives (compare 48, 49 ; 59, 64).

Alkylation of the amino groups produces a similar result in the case of the triphenyl methane dyes, but has the reverse effect in the acridine series, since the tetramethyl and tetraethyl derivatives of 2.7 diaminoacridine methochloride are somewhat weaker than the unsubstituted compound.

Acetylamino Group.—Acetylation of the amino group in the benzene nucleus reduces the antiseptic action (compare Nos. 1, 86 ; 6, 87 ; 11, 90 ; 45, 94). It may be observed that these substances, which, like the unsubstituted compound (83), have only a weak action, are characterized by the absence of the basic group from the alternate linkage system; and the same consideration applies to substances 84, 85. The diacetyl derivatives (88, 89) are so little soluble in peptone water that no significance attaches to the results, but they are more soluble in serum, and in this medium their antiseptic action is very weak.

Substitution in the Quinoline Nucleus.

The introduction of a further condensed nucleus, *i.e.*, the formation of beta-naphtho-quinoline derivatives, slightly increases the antiseptic action in the case of the styryl compounds (compare Nos. 5, 1; 17, 14). The corresponding anil shows a marked increase (52 compared with 47), and is an exceedingly powerful antiseptic against both *S. aureus* and *B. coli* in peptone water and serum. On the other hand, the simpler pyridine derivative (13) is weaker in its antiseptic action than the corresponding unsubstituted quinoline compound (15).

In the dimethylamino styryl series, the introduction of 6 methyl and 6 ethoxy groups increases the potency, especially for Staphylococcus, but the 6 methoxy has little effect (compare 16 with 14; 19, 18 with 15). In the case of the less effective *p*-amino styryl compound (1) substitution by methyl (4) does not give rise to any marked change.

In the dimethylamino anil series, the introduction of 6 methyl (48), 6·8 dimethyl (51), 6 methoxy (53), and 6 ethoxy (54), has no pronounced effect (compare with 47); and the same is true of the presence of a methyl group in the *p*-amino anil (compare 42, 41). The 6.7 methylene-dioxy derivatives of both series are somewhat less effective on both organisms in watery medium, but show little or no change in serum (compare 20, 14; 55, 47):

Primary Amino Groups.—The effect of a primary amino group in the quinoline nucleus is somewhat indeterminate, the change in potency being in no case very great. Thus, whereas Nos. 6 and 21 are very slightly more active than 1 and 14, respectively, although only for Staphylococcus in serum, the corresponding anils 43 and 56 do not differ from the unsubstituted compounds 41 and 47, respectively. Comparison of the acetylamino styryls 87, 86 shows a slight weakening effect of the 6-amino group. The 7-amino compound (44) does not differ markedly from the 6-amino isomer (43).

Tertiary Amino Groups.—Replacement of the 6 primary amino group by a 6 dimethylamino group leads to very little increase in potency, especially where the primary compound is fairly active already (compare 6, 11; 21, 37; 43, 64;

56, 75). In the case of 87, 90, the activity of the latter may be explained by its greater solubility.

Acyl-Amino Groups.—It has already been observed that acetylation of an amino group in the benzene nucleus lowers the antiseptic activity. On the other hand, as a result of acylation of the 6 amino group in the quinoline nucleus, which group is independent of the alternate linkage system, the tendency is rather in the direction of increased activity. Some of the acyl derivatives of 2 (*p*-dimethylamino anil) 6 amino quinoline methochloride are, in fact, amongst the most potent antiseptics known in their action on *B. coli*; this is apparent from the comparison of 56 with 59, 65, 66, 67, 68, in which the acyl group varies from acetyl through the normal fatty acid series up to *n*-caproyl. This effect is also seen with the corresponding methiodides. Thus 61 is more than twenty times as potent as 57. The formyl group (58) is apparently a depressant.

In a similar series of acylamino styryl compounds (22, 24, 27, 28, 29, 30), apart from the weaker formyl derivative, there is little change in activity. Their relative insolubility prevents a satisfactory estimation of their action on *B. coli* in watery medium.

In the case of the acetyl and propionyl derivatives of 2 (*p*-amino styryl) 6 amino quinoline methochloride (8, 9) there is a distinct reduction in potency (compare 6), and in the corresponding acetyl-amino anil (45) little effect beyond a slightly increased activity of the acyl compound in serum for *B. coli* (compare 43). The solubility factor doubtless affects the result obtained with 8, as it will be noted that this substance is considerably less soluble in both media than the more active amino compound 6. This view receives some support from the results obtained with the corresponding 7 acetylamino compound (10), which is at once more soluble and more active than 8. This fact should be considered in conjunction with the observations that the 6 and 7 acetylamino compounds 25 and 36 (methosulphates) are practically equal in antiseptic power, as well as in solubility, and that the methochlorides of the corresponding anil compounds are also practically equal (59, 74).

The effect of other acyl radicals has also been examined. In the dimethylamino styryl series, the diethyl-acetyl derivative (31) is of the same order of activity as the normal fatty acyl compounds, the brom-diethyl-acetyl compound (32) is more powerful, but chloracetyl (33), bromacetyl (34), and trichloracetyl (35) groups appear to reduce the antiseptic action. In the corresponding anil series, the diethyl-acetyl group (69) has again a similar effect to the normal fatty acyl groups, and the 6 brom-diethyl-acetyl compound (70) is almost equal to the others of the series, but the chloracetyl (71), bromacetyl (72), and benzoyl

(73) amino derivatives are less active against *Staphylococcus* in serum. As in the styryl series, this effect is possibly due to the more acidic character of the acyl radicals.

Acidic Groups.—Acidic groups in the 6 position generally appear to diminish antiseptic action. Although chlorine (77) does not produce any change, bromine (78) and the nitro group (79) cause a reduction in potency which is especially marked in serum (compare with 47). The similar tendency of the halogens when introduced into the acyl-amino group has already been pointed out. The styryl compound containing a nitro group is too insoluble for satisfactory testing.

Sulphonation.

Although sulphonic derivatives of known orientation have not been prepared, owing to the difficulty of forming quaternary salts of such substances, some of the styryl and anil methochlorides have been sulphonated, and have yielded products of increased solubility in water. In the styryl series, 12 and 39 are more active than the unsulphonated compounds 8 and 24 respectively, whereas 81, a product of sulphonation of anil 59, showed practically unchanged potency.

Azo-Groups.

The azo-dyestuffs 30 and 80, derivatives respectively of styryl 21 and anil 56 with 1 amino 8 naphthol 3·6 disulphonic acid (H-acid), showed practically no antiseptic action.

Position of the Styryl and Anil Groups with Respect to the Quinoline Nucleus.

4 *p*-Dimethylamino-styryl and anil compounds (40 and 82), *i.e.*, derivatives of lepidine, were both found to be much less active than the corresponding quinaldine compounds 16 and 48. A change in the length and formation of the alternate linkage system is here involved, and it is noteworthy that the anil is distinctly weaker than the styryl derivative, in contrast with the rule in the 2-styryl and anil series.

Changes in the Quaternary Grouping of the Quinoline Nitrogen.

A comparison of the hydrochloride and the methochloride of the same base, 2 *p*-dimethylaminostyryl 6 acetylaminoquinoline (23, 24), shows that the latter is much the more active. In the case of the corresponding *p*-amino-styryls (7, 8), although the hydrochloride is inactive, the relative weakness of the methochloride prevents any effective comparison. The same effect, however, was often observed in the acridine series.

Ethyl quaternary salts (26, 63) do not differ appreciably in activity from the corresponding methyl derivatives (25, 62).

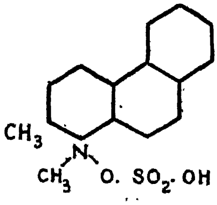
The effect of varying the acid radical has been tested in several instances. In the case of 2 (*p*-dimethylamino anil) 6 acetyl-amino-quinoline, the methochloride (59), methobromide (60), methiodide (61), and methosulphate (62) showed no marked difference. The same may be said of compounds 49, 50 and 75, 76. But in some instances, a striking difference was observed, *e.g.*, with the methochloride 56 and methiodide 57 of 2 (*p*-dimethylaminoanil)-6 aminoquinoline, the latter being much the weaker antiseptic, and much less soluble. The difference in potency may therefore be due directly to this physical factor, although no obvious precipitation occurs within the range of sterilising concentrations. It is also noteworthy that, as tested by subcutaneous injection in mice, the tolerated dose of the methiodide was about three times greater than that of the methochloride. It may be that low solubility retards diffusion, an effect which would apply to other sparingly soluble compounds.

Chemical Section.

Intermediate Compounds.—Many of the intermediate compounds involved in the preparation of the styryl and anil quinolines have been described in a former paper ('Roy. Soc. Proc.,' B, vol. 96, p. 317 (1924)), further reference to these being unnecessary.

β-*Naphtho-quinaldine* was prepared from β-naphthylamine according to a modification (private communication) of the method of Döbner and Miller. It was obtained in the form of colourless needles melting at 80–82°. The yield amounted to 24 per cent. of the theory.

β-*Naphtho-quinaldine methochloride.*—The methosulphate was first prepared by the direct action of dimethyl sulphate. It was boiled with concentrated hydrochloric acid and the solution poured into alcohol. The colourless crystalline compound which separated was apparently the acid methosulphate.



The methochloride was obtained by treating the boiling aqueous solution of the acid methosulphate with the theoretical quantity of barium chloride, and evaporating the filtered solution to dryness.

β-*Naphtho-quinaldine metho-p-toluene sulphonate.*—This was obtained by the action of methyl *p*-toluene sulphonate in nitrobenzene solution at 140°. The product was washed with ether and recrystallised from alcohol. It is easily soluble in water.

6.8-*Dimethyl quinaldine* was obtained by a modification of the process of Panajotow ('Ber.,' vol. 20, p. 32 (1887)), the product, after separation by distillation in steam, being further purified by treatment with nitrous acid. The base was dissolved in dilute hydrochloric acid, cooled, and a slight excess of sodium nitrite added. The resulting nitrosamines were removed by extraction with ether, and the aqueous layer, after boiling for a few minutes, was made alkaline and distilled in steam. The purified base was again distilled, being collected at 265–70°. The yellow oil solidified on standing, and was pressed on a porous plate, yielding colourless crystals melting at 43–6°. By allowing the chloroform solution to evaporate slowly, rhombohedral crystals, melting at 44·5°–46·5°, were obtained.

6.8-*Dimethyl quinaldine methochloride* was made in the same way as the preceding methochloride.

6-*Chloro-quinaldine* was prepared from *p*-chloroaniline by the ordinary Döbner-Miller reaction. The diluted reaction mixture was filtered from some impurity, and the product separated by distillation in steam. No tar was formed. Purification was effected by treatment with nitrous acid, and the final product was recrystallised from dilute alcohol, the yield being about 36 per cent. of the theory. After three successive crystallisations, the substance melted at 93–6°. It separated in colourless plates, or clusters of fine needles.

6-*Chloro-quinaldine methochloride* was obtained in the same way as the β-naphtho-quinaldine compound. Crude dimethyl sulphate was used, without any solvent, as in the case of the nitro-derivative, described below.

6-*Bromo-quinaldine.*—*p*-Bromo-aniline hydrochloride was used in this preparation, the quantity of hydrochloric acid being proportionately reduced. The method was the same as that employed for 6-chloro-quinaldine, the product being finally pressed on a porous plate. It crystallises from alcohol in colourless prisms, melting at 101–2°. The yield was 33 per cent. of the theory.

6-*Bromo-quinaldine methochloride* was obtained from the methosulphate as in the preceding case. The methosulphate was made by heating the base in nitrobenzene solution for half an hour on the water-bath with a slight excess of dimethyl sulphate. The yield was 84 per cent. of the theory.

6-*Nitro-quinaldine methochloride.*—6-Nitro-quinaldine exhibited unusual behaviour when treated with purified dimethyl sulphate. In nitrobenzene solution combination appeared to be slow, whereas a deep red or purple coloration was invariably observed. With methyl *p*-toluene sulphonate a still more rapid formation of this intense colour was noticed, and in no case, with or without a solvent, could a crystalline product be isolated. The methosulphate was ultimately obtained by heating the base for 15 minutes at 140° with the theoretical quantity of *crude* commercial dimethyl sulphate. The deep coloration did not appear, apparently owing to the acidity of the dimethyl sulphate, and a hard brownish glassy mass was formed, which was boiled with concentrated hydrochloric acid and barium chloride. The filtered solution was evaporated to dryness, and the residue extracted with boiling alcohol with the addition of some charcoal. On concentration of the alcoholic solution small colourless crystals were obtained.

0·2977 gm. gave 0·1781 grm. AgCl

Cl = 14·80 per cent.

$C_{11}H_{11}O_2N_2Cl$ req. Cl = 14·86 per cent.

6-*Formyl-amino-quinaldine*.—6-Amino-quinaldine was boiled for two hours with about twice its weight of formic acid (90 per cent.). The mixture was diluted, and made alkaline with ammonia. The oily product soon solidified, and was filtered off and recrystallised from hot water. The anhydrous substance melts at 131–132°.

6-*Formyl-amino-quinaldine methiodide* was prepared from the base by the action of methyl iodide in nitrobenzene solution, as described in the former paper for the acetyl derivative. The yield was 93 per cent. of the theory.

6-*Acetyl-amino-quinaldine methochloride*.—The methosulphate was first obtained by the action at 100° of dimethyl sulphate on the nitro-benzene solution of the base. The product was washed thoroughly with ether, and dissolved in a small quantity of water, in which it was extremely soluble. On the addition of saturated brine the methochloride separated out almost completely, and was filtered, washed with brine, and finally dried on the water bath. This product was free from sulphur, but contained about 10 per cent. of salt. No disadvantage, however, was subsequently experienced on this account. A yield of 84 per cent. of the theory of (pure) methochloride was obtained from the base.

It was curious that the methochloride could be salted out in this way only when the acyl group was acetyl or substituted acetyl, although the methiodide could always be caused to separate by the addition of potassium iodide.

6-*Acetyl-amino-quinaldine methobromide* was prepared from the metho-sulphate in the same way by salting out with potassium bromide.

6-*Propionyl-amino-quinaldine.*—6-Amino-quinaldine was dissolved in propionic acid on the water bath, and excess of propionyl chloride added. The mixture almost immediately set solid owing to the separation of 6-propionyl-amino-quinaldine hydrochloride. Water was added to the cooled mixture, the whole going into solution. The mixture was made alkaline with ammonia, and the product filtered and recrystallised from boiling water, from which it separated in long silky needles. This was a hydrate, which melted rather indefinitely at about 80°, lost water at 100°, and solidified again, melting finally at 146–148°. The hydrate also lost water when kept for 12 hours in a vacuum desiccator at the ordinary temperature. The yield was 70 per cent. of the theory.

6-*Propionyl-amino-quinaldine methiodide* was obtained in the same way as the acetyl derivative, and formed pale yellow prisms, sparingly soluble in alcohol, easily soluble in water, or in alcohol containing a little water.

The remaining acyl-amino-quinaldines were all prepared in the same way as the propionyl compound, using the appropriate acid chloride. Acetic acid, however, was used as solvent, and when the hydrochloride of the acyl compound separated out, it was found desirable to filter this off, before converting to the base. In some cases no such separation took place, and the whole mixture was therefore neutralised.

The acyl-amino-quinaldines are colourless substances, soluble in alcohol, chloroform, and benzene. They are almost insoluble in cold water, and the solubility in hot water decreases with increasing molecular weight. The various compounds prepared crystallise and melt as follows :—

	Crystallisation.	m.p. of Anhydrous Substance.
6-*Butyryl-amino-quinaldine* ..	Needles, from water (or dilute alcohol)	134–5°
6-*Valeryl-amino-quinaldine*	Needles, from water (or dilute alcohol)	156–8°
6-*n-Caproyl-amino-quinaldine*	Needles, from water (or dilute alcohol)	166–7°
6-*Diethyl-acetyl-amino-quinaldine.*	Flat prisms, from water (or dilute alcohol)	165–6°

	Crystallisation.	m.p. of Anhydrous Substance.
6-*Chloracetyl-amino-quinaldine*	Needles, from water (or dilute alcohol)	164–6°
6-*Bromacetyl-amino-quinaldine*	Microscopic crystals, from alcohol	161–3° (with decomposition).
6-*Trichloracetylamino - quinaldine.*	Plates, from dilute alcohol ..	167–8°
6-*Bromdiethyl - acetyl - amino-quinaldine.*	Flat prisms, from dilute alcohol	122–3°
6-*Benzoyl-amino-quinaldine* ..	Prisms, from dilute alcohol ..	176–7°

Some of these substances, like the propionyl compound, form low-melting hydrates. The chloracetyl and bromacetyl derivatives, together with their methochlorides, exert an extremely powerful irritant action upon the skin. The methochloride of the benzoyl compound could be salted out from the solution of the methosulphate, as in the case of the acetyl derivative.

6-*Dimethylamino-quinaldine methiodide.*—10·4 gms. 6-amino-quinaldine and 28 gms. methyl iodide were boiled under reflux for five hours with 200 c.c. water containing 7 gms. sodium carbonate. The clear deep-orange solution deposited orange-yellow crystals on cooling. These were recrystallised from hot water.

0·5060 gm. gave 0·3619 gm. AgI.

I = 38·66 per cent.

$C_{13}H_{17}N_2I$ requires I = 38·69 per cent.

Methyl *p*-toluene sulphonate was also tried as methylating agent, thus avoiding the use of the volatile methyl iodide. Methylation was very rapid, and the methiodide was obtained from the resulting clear solution by the addition of potassium iodide. The methochloride, however, did not separate on salting with sodium chloride.

6-*Amino-quinaldine methiodide.*—6-acetyl-amino-quinaldine methiodide was boiled with strong hydrochloric acid for 2 hours. The mixture was cooled and most of the acid carefully neutralised with ammonia. The product separated in yellow needles, which were recrystallised from aqueous alcohol containing some potassium iodide.

0·3094 gm. gave 0·2414 gm. AgI.

I = 42·17 per cent.

$C_{11}H_{13}N_2I$ requires I = 42·32 per cent.

6-Amino-quinaldine methochloride.—6-acetyl-amino-quinaldine was heated with the theoretical quantity of methyl *p*-toluene sulphonate in nitrobenzene solution at 130-140° for 1½ hours. The yellow crystalline product was recrystallised from alcohol, forming yellow prisms.

0·5185 gm. gave 34·6 c.c. N. at 13° and 759 mm.

N = 7·86 per cent.

$C_{20}H_{22}O_4N_2S$ requires N = 7·26 per cent.

The metho *p*-toluene sulphonate was boiled with strong hydrochloric acid diluted with an equal volume of water. Ammonia was added to the cooled solution till the acid was almost neutralised, the resulting crystalline precipitate of 6-amino-quinaldine methochloride being recrystallised from alcohol containing a little water; in which, like most of the quinaldine quaternary halides, it was much more soluble than in pure alcohol.

6.7-*Methylene-dioxy quinaldine* was made according to the method of Borsche and Quast ('Ber.,' vol. 52, p. 432 (1919)). It was purified by distillation under reduced pressure, being collected at 190–2° at about 10 mm. After cooling, the substance formed an almost colourless crystalline mass, melting at 146-8°. It was converted to the methosulphate in nitrobenzene solution in the usual way.

6-*Methyl lepidine and its methiodide.*—6-Methyl lepidine was made from *p*-toluidine according to the method of Mikeska ('Jl. Amer. Chem. Soc.,' vol. 42, p. 2396 (1920)). The base was separated by distillation in steam, instead of by extraction with ether. The methiodide was made by heating directly with methyl iodide on the water-bath. It crystallised from alcohol in yellow needles.

p-Amino-benzaldehyde was prepared by the method described by Geigy and Co. ('D.R.P. 86874, Friedländer,' IV, 136). The oily product was dissolved in alcohol, filtered quickly from a little insoluble matter, and used immediately for condensation with the quinaldine derivatives.

Styryl Compounds.—The *p*-dimethylamino-styryl compounds were all prepared as described by Mills and Pope ('Trans. Chem. Soc.,' vol. 121, p. 946 (1922)), and by the present authors ('Brit. Med. Journ.,' vol. II, p. 326 (1923)). It was usually necessary to add water to the alcohol in sufficient quantity to effect solution of the quinaldine quaternary compound, thus avoiding the use of a very large bulk of alcohol.

The methochlorides of the dimethylamino-styryl quinolines crystallise from alcohol in dark red prisms or needles, usually, though not invariably, showing a green reflex. The solution in alcohol is bright bluish red in colour, the aqueous solutions being orange-red in appearance. The solubility usually decreases with increasing size of the quinoline molecule.

2 (*p-Dimethylamino-styryl*) 6-*nitro-quinoline methochloride.*—Comment has already been made upon the unusual activity of 6-nitro-quinaldine methochloride in undergoing condensation, and it was found that condensation with dimethylamino benzaldehyde took place without the addition of piperidine on boiling the alcoholic solutions for 15-20 minutes. The product separated in prisms showing a brilliant green reflex. It is sparingly soluble in alcohol and water. The alcoholic solution is of a pure blue colour.

0·1821 gm. gave 18·5 c.c. N at 19° and 754 mm.

N = 11·64 per cent.

$C_{20}H_{20}O_2N_3Cl$ requires N = 11·37 per cent.

2 (*p-Dimethylamino-styryl*) 6-*amino-quinoline methochloride* (21).—*p*-Dimethylamino-benzaldehyde was condensed with 6-acetylamino-quinaldine metho-*p*-toluene sulphonate. The product, which separated in prisms showing a bright green reflex, was boiled for 2 hours with concentrated hydrochloric acid. The solution was diluted and neutralised with ammonia, the resulting precipitate being filtered off and recrystallised from alcohol. This substance was found to be the metho *p*-toluene sulphonate of the amino compound and not the methochloride. The latter was obtained, however, by boiling the former with an aqueous solution of sodium chloride, and formed small prisms showing a bright green reflex. It was filtered and washed with water.

0·2313 gm. gave 27·0 c.c. N at 20° and 741 mm.

N = 13·02 per cent.

0·1183 gm. gave 0·0486 gm. AgCl

Cl = 10·17 per cent.

$C_{20}H_{22}N_3Cl$ requires N = 12·37 per cent.

Cl = 10·44 per cent.

2 (*p-Dimethylamino-styryl*) 6 *chloracetylamino-quinoline methochloride* (33).—It was found that dimethylamino benzaldehyde and 6-chloracetylamino-quinaldine methochloride did not undergo the usual condensation in alcoholic solution in presence of piperidine. This was probably due to condensation of the piperidine with the chloracetyl group, the reaction proceeding more rapidly than the styryl condensation. The corresponding anil, it may be noted, could

be prepared in the normal manner, no doubt owing to the greater rapidity of the anil condensation, when compared with styryl formation, a characteristic frequently noted in preparing the two series of compounds.

The styryl derivative was obtained by the action of chloracetyl chloride on 2 (*p*-dimethylamino-styryl) 6 amino-quinoline methochloride. The latter was suspended in glacial acetic acid and heated at 100°, excess of the acid chloride being added. On shaking vigorously, a clear pale yellow solution was obtained. The hydrochloride of the product separated from the cooled mixture in pale yellow crystals, which were filtered off, washed with acetic acid, and mixed with a large volume of water. The hydrochloride was immediately hydrolysed, and the dark red base deposited. It was filtered, and stirred with very dilute hydrochloric acid, to remove any unchanged material, and washed with water. It was finally boiled with alcohol, and formed large crystals exhibiting the usual green lustre. The substance is very sparingly soluble in water.

2 (*p-Dimethylamino-styryl*) 6 *bromacetylamino-quinoline methochloride* (34).—This was prepared in the same way as the preceding compound, using bromacetyl chloride. Bromacetyl bromide gave unsatisfactory results, yielding a tarry product. The compound is only slightly soluble in cold water, but dissolves to some extent on heating.

0·2853 gm. gave 23·6 c.c. N at 8° and 742 mm.

N = 9·75 per cent.

$C_{22}H_{23}ON_3ClBr$ requires N = 9·12 per cent.

2 (*p-Dimethylamino-styryl*) 6 *trichloracetylamino-quinoline methochloride* (35).—This substance was prepared in the same way as the two preceding compounds, using trichloracetyl chloride. As the hydrochloride did not separate, however, from the acetic acid solution, the whole mixture was diluted, when the dark-coloured product was precipitated. It was filtered off, washed with water, and recrystallised from alcohol, separating in lustrous green prisms, sparingly soluble in water.

2 (*p-Dimethylamino-styryl*) 6 *bromdiethylacetylamino-quinoline methochloride* (32) was similarly obtained by the use of bromdiethylacetyl chloride. The hydrochloride in this case separated in oily form, but a solid product was obtained on dilution of the whole mixture.

Sulphonation products (39, 12) of 2 (*p*-dimethylamino-styryl) 6 acetylamino-quinoline methochloride, and also of 2 (*p*-amino-styryl) 6 acetylamino-quinoline methochloride were prepared by warming these substances with 6–8 times their weight of 100 per cent. sulphuric acid for 3–4 hours on the water-bath. The mixture was allowed to stand at room temperature for 12–16 hours, and was then poured on to ice. The acid solution was neutralised as quickly as possible with ammonia, and a little salt added to assist separation of the product, which was filtered off and washed with a small quantity of cold water. These sulphonation products were considerably more soluble in water than the parent styryl compounds.

2 *(p-Dimethylamino-styryl) 6-azo-quinoline methochloride-H-acid* (38).—6-Amino-quinaldine methochloride was diazotised and coupled with H-acid in sodium carbonate solution, the resulting azo dyestuff being salted out in the usual manner. It was freed from salt as far as possible by treatment with a small quantity of cold water. This substance was condensed in aqueous alcoholic solution with *p*-dimethylamino benzaldehyde in the ordinary way, the product separating more readily on the further addition of alcohol to the mixture. It is extremely soluble in water.

4 *(p-Dimethylamino-styryl) 6 methyl-quinoline methochloride* (40).—*p*-Dimethyl-amino-benzaldehyde was condensed with 6-methyl lepidine methiodide. The crystalline product was boiled with an alcoholic suspension of silver chloride, and the filtered solution evaporated to dryness. The product was finally crystallised from alcohol.

2 *(p-Amino-styryl) 6 acetylamino-quinoline methochloride* (8) was prepared in the same way as the dimethylamino styryl compounds, using the filtered alcoholic solution of freshly made *p*-amino-benzaldehyde. The product separated on cooling in needles or prisms showing the usual green reflex.

0·2231 gm. gave 23·8 ccs. N. at 21° and 732 mm.

N = 11·76 per cent.

$C_{20}H_{20}ON_3Cl$ requires N = 11·88 per cent.

2 *(p-Amino-styryl) 6 dimethylamino-quinoline methiodide* was obtained in the same way as the preceding substance from 6-dimethylamino quinoline methiodide. It formed minute needles exhibiting a very dark bronze green reflex.

0·2423 gm. gave 20·2 c.c. N at 9° and 738 mm.

N = 9·74 per cent.

$C_{20}H_{22}N_3I$ requires N = 9·75 per cent.

The methochloride (11) was prepared by boiling the alcoholic solution of the methiodide with excess of silver chloride.

The alcoholic and aqueous solutions of these amino-styryl derivatives are redder in colour than the corresponding dimethylamino styryl compounds.

2 (*p-Amino-styryl*) 6 *amino-quinoline methochloride* (6) was prepared by hydrolysis of the corresponding acetyl amino styryl compound (87) with concentrated hydrochloric acid. The product was precipitated by neutralisation with ammonia, and was recrystallised from alcohol.

Amino-styryl 6 *methyl-quinoline methochlorides.*—The appropriate nitrobenzaldehyde was condensed with the theoretical quantity of *p*-toluquinaldine by heating the substances together at 100° in presence of a little zinc chloride until the mass became completely solid.

The resulting compounds, after purification, were reduced with iron and acetic acid, and isolated as hydrochloride, as described by Mills and Evans (' Trans. Chem. Soc.,' vol. 117, p. 1038 (1920)). Both the nitro- and amino-compounds are insoluble in water.

The acetyl derivative was next prepared by boiling the amino compound with acetic anhydride, a little fused sodium acetate being added. The product was recrystallised and combined in nitrobenzene solution with dimethyl sulphate or methyl *p*-toluene sulphonate, and the resulting quaternary compound boiled with moderately strong hydrochloric acid. The product separated as hydrochloride, which was filtered, dissolved in water and treated with ammonia, when the final product separated. Some sodium chloride was usually added to the mixture before neutralisation.

2 (*o-Nitro-styryl*) 6 *methyl quinoline* was recrystallised from xylene, and washed with acetone. It forms colourless prisms melting at 160–1°.

2 (*o-Amino-styryl*) 6 *methyl quinoline* crystallises from alcohol in pale yellow prisms or needles melting at 178–9°.

2 (*o-Acetyl-amino-styryl*) 6 *methyl quinoline* separates from alcohol in pale yellow crystals of melting point 188–190°. It was combined with dimethyl sulphate at 130°.

2 (*o-Amino-styryl*) 6 *methyl quinoline methochloride* (2) was purified by dissolving in hot water and filtering from a little insoluble residue. The filtrate was made slightly acid, with hydrochloric acid, heated to boiling, and a little salt added. The methochloride separated on cooling in red needles. It was finally washed with a little cold water, in which it is rather sparingly soluble.

2 (*m-Nitro-styryl*) 6 *methyl quinoline* crystallises from xylene or acetone in yellow prisms or plates melting at 198–200°.

2 (*m-Amino-styryl*) 6 *methyl quinoline* separates from aqueous alcohol in colourless needles melting at 159–161°.

2 (*m-Acetyl-amino-styryl*) 6 *methyl quinoline* melts at 167–8°. It crystallises

from dilute alcohol in colourless needles. It was combined with methyl *p*-toluene sulphonate at 130–140°, the product being hydrolysed by means of a mixture of equal volumes of strong hydrochloric acid and water.

2 (*m-Amino-styryl*) 6 *methyl quinoline methochloride* (3) was purified in the same way as the ortho compound. It forms fine orange-red needles, more soluble in water than the ortho derivative.

2 (*p-Nitro-styryl*) 6 *methyl quinoline* forms yellow needles (from xylene) of melting point 187–8°.

2 (*p-Amino-styryl*) 6 *methyl quinoline* melts at 190–1°. It separates from alcohol in small yellow needles or prisms.

2 (*p-Acetyl-amino-styryl*) 6 *methyl quinoline* is very sparingly soluble in hot alcohol, and after boiling with that solvent, remained as a colourless crystalline residue melting at 247–250°, which was converted to the methosulphate.

2 (*p-Amino-styryl*) 6 *methyl quinoline methochloride* (4) is more soluble in water than the ortho compound. It was purified by recrystallisation from methyl alcohol, and formed beautiful large prisms showing a steel blue metallic reflex.

The *p*-acetylamino styryl compounds were prepared by the condensation in aqueous alcoholic solution, in presence of piperidine, of *p*-acetylamino benzaldehyde with the appropriate quinaldine derivative. Condensation proceeded rapidly, the product usually separating from the hot solution. The crystals were filtered and washed thoroughly with alcohol.

2 (*p-Acetyl-amino-styryl*) 6 *acetylamino-quinoline methochloride* (88) was obtained in this way with a yield of 82 per cent. of the theory.

0·2712 gm. gave 24·0 c.c. N at 9° and 759 mm.

N = 10·64 per cent.

$C_{22}H_{22}O_2N_3Cl$ requires N = 10·62 per cent.

2 (3.4 *Dihydroxy-styryl*) *quinoline methosulphate* (84).

Protocatechuic aldehyde and quinaldine condensed easily when heated together in equimolecular proportions at 110° for 5 hours. The product was purified by crystallisation from alcohol, and was converted to the methosulphate in nitrobenzene solution at 150°.

The latter forms pale orange crystals, easily soluble in water, giving a yellow solution.

2 (3.4 *Methylene-dioxy-styryl*) *quinoline methosulphate* (85).

Piperonal and quinaldine were heated together in equimolecular quantities for several hours at 140–150°, and the solid product crystallised from alcohol. The methosulphate, prepared in the usual way, forms beautiful orange-red prisms, easily soluble in warm water.

2 *Styryl* 6 *methyl quinoline methochloride* (83).

Equivalent proportions of benzaldehyde and 6-methyl quinaldine were heated for 45 hours at 100° in a sealed tube, together with a fragment of zinc chloride. The resulting solid mass was recrystallised from alcohol, and yielded colourless prisms melting at 136–7°.

The methosulphate was obtained by heating the base with dimethyl sulphate for 15 minutes at 100° in toluene solution. The product rapidly separated in bright lemon-yellow crystals, soluble in water. These were boiled for half an hour with strong hydrochloric acid, and the yellow crystals which separated on cooling filtered off. Purification was effected by dissolving in water, filtering, and reprecipitating by the addition of a little hydrochloric acid.

Anil Compounds.

The dimethylamino anils were prepared as described by the present authors (' Journ. Path. and Bact.,' vol. 27, pp. 121–2 (1924)). It was frequently necessary to allow the reaction to proceed at a moderate temperature, *e.g.*, 50–60°, rather than to heat the alcoholic solution rapidly to boiling point. In some instances, where a methochloride was required, it was found that the condensation and separation of the product proceeded more satisfactorily with the methiodide of the quinaldine derivative than with the methochloride. In such cases, therefore, the methiodide of the anil was first made, and was converted to the methochloride by boiling in alcoholic or aqueous alcoholic solution with silver chloride.

The anils give solutions in alcohol or water of a much bluer shade than the corresponding styryl compounds. They usually crystallise well from alcohol in prisms or needles showing a characteristic green reflex. Unlike the styryl compounds, they are decomposed by weak mineral acid solutions.

p-Nitroso-dimethylaniline was found to condense quite satisfactorily with amino quinaldine quaternary salts without interference with the free amino group, 2 (*p*-dimethylamino-anil) 6 amino-quinoline methochloride, for example, being made by condensation with 6-amino-quinaldine methochloride. The *p*-amino-anils were also obtained by the same method, using *p*-nitroso-aniline in place of *p*-nitroso-dimethylaniline. They yield solutions of a redder shade than the dimethylamino compounds. 2-Anil compounds were made in the same way by the use of nitroso benzene.

2 (*p-Dimethylamino-Anil*) 6 *Nitro-Quinoline Methochloride* (79).

6-Nitro-quinaldine methochloride condensed with *p*-nitroso-dimethylaniline, without the addition of piperidine, even more easily than with dimethylamino-benzaldehyde. It was merely necessary to mix the hot alcoholic solutions of the two reagents to obtain a thick crystalline precipitate of the product, which was filtered off and recrystallised from alcohol. It formed small prisms exhibiting a brilliant green reflex and giving a pure blue solution in alcohol. The crystals apparently contained alcohol of crystallisation.

0·1994 gm. gave 24·5 c.c. N at 18·5° and 744·5 mm.

N = 13·87 per cent.

$C_{19}H_{19}O_2N_4Cl + C_2H_5OH$ requires N = 13·45 per cent.

The *sulphonation product* (81) of 2 (*p*-dimethylamino-anil) 6 acetylamino-quinoline methochloride was obtained in the same way as the corresponding styryl compound.

2 (*p-Dimethylamino-anil*) 6 *azo-quinoline methochloride*-H *acid* (80) was made in the same way as the corresponding styryl compound. It was precipitated with alcohol, and is very soluble in water, giving a blue solution.

2 (*p-Acetylamino-anil*) 6 *acetylamino-quinoline methochloride* (94) was prepared by boiling 2 (*p*-amino-anil) 6 acetylamino-quinoline methochloride for 15–20 minutes with 10–12 times its weight of acetic anhydride. The product separated from the hot solution in brownish-yellow crystals, which were filtered off and washed with fresh acetic anhydride, followed by a little alcohol.

0·1940 gm. gave 24·4 c.c. N at 19° and 751 mm.

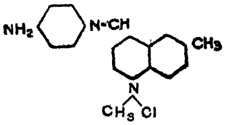
N = 14·35 per cent.

$C_{21}H_{21}O_2N_4Cl$ requires N = 14·13 per cent.

In the following tables (Tables II–VI) the figures representing antiseptic potencies are the reciprocals, divided by 1000, of the dilutions which produce the effects indicated. In the columns headed *precipitation* the figures, similarly converted, record the highest dilution, within the range investigated, at which precipitation occurs in peptone water (P) and serum (S) respectively. The sign — indicates absence of precipitation.

Names of substances are expressed thus :—St = styryl ; A = anil ; P = pyridine ; Q = quinoline. (MeCl), (MeI), (MeS), (EtS) = methochloride, methiodide, methosulphate, ethosulphate. Substituent groups are denoted by their chemical symbols, position in the quinoline nucleus being indicated by a figure above the group, and in the benzene nucleus by *o*, *m*, or *p*.

Thus, $p.NH_2\overset{4}{A}.Q.\overset{6}{CH_3}$ (MeCl) represents



and $o.NH_2\overset{2}{S}t.Q.\overset{6}{C}H_3$ (MeCl) denotes

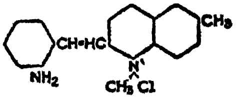


Table II.—Amino-Styryl Compounds.

No.	Substance.	Staphylococcus Aureus.						B. Coli.						Precipitation.	
		Peptone water.			Serum.			Peptone water.			Serum.			P.	S.
		+	±	−	+	±	−	+	±	−	+	±	−		
1	$p\text{-}NH_2\,\overset{2}{St}\,.\,Q\,.\,(Me\,Cl)$	100	40	20	40	—	20	10	4	2	40	20	10	—	—
2	$o\text{-}NH_2\,\overset{2}{St}\,.\,Q\,.\,\overset{6}{CH_3}\,(Me\,Cl)$	40	10	4	2	—	?	4	2	?	4	2	?	2	—
3	$m\text{-}NH_2\,\overset{2}{St}\,.\,Q\,.\,\overset{6}{CH_3}\,(Me\,Cl)$	40	10	4	20	10	4	10	4	1	20	10	4	—	—
4	$p\text{-}NH_2\,\overset{2}{St}\,.\,Q\,.\,\overset{6}{CH_3}\,(Me\,Cl)$	100	—	40	100	40	20	20	10	1	40	20	10	4	—
5	$p\text{-}NH_2\,\overset{2}{St}\,.\,\beta\text{ naphtho }Q\,.\,(Me\,Cl)$	400	200	100	200	—	100	2	—	?	200	100	40	10	2
6	$p\text{-}NH_2\,\overset{2}{St}\,.\,Q\,.\,\overset{6}{NH_2}\,(Me\,Cl)$	200	100	40	200	—	100	40	20	4	200	100	20	—	—
7	$p\text{-}NH_2\,\overset{2}{St}\,.\,Q\,.\,\overbrace{NH\,.\,CO\,.\,CH_3}^{6}\,(H\,Cl)$	4	2	?	2	—	?	2	—	?	2	—	?	20	8
8	$p\text{-}NH_2\,\overset{2}{St}\,.\,Q\,.\,\overbrace{NH\,.\,COCH_3}^{6}\,(Me\,Cl)$	2	1	?	40	20	4	1	—	?	1	—	?	10	10

9	p-$NH_2 \overset{2}{St} . Q . \overbrace{NH . CO\,C_2H_5}^{6}$ (Me Cl)	....	40	20	?	100	40	20	1	—	?	20	10	4	10	4
10	p-$NH_2 \overset{2}{St} . Q . \overbrace{NH . CO\,CH_3}^{7}$ (Me Cl)	....	40	20	?	100	40	20	1	—	?	20	—	10	4	—
11	p-$NH_2 \overset{2}{St} . Q . \overset{6}{N} (CH_3)_2$ (Me Cl)	....	400	200	100	400	200	100	10	—	4	200	—	100	—	—
12	p-$NH_2 \overset{2}{St} . Q . \overbrace{NH . COCH_3}^{6}$ (Me Cl) .... sulphonated	....	400	200	40	200	100	40	200	100	10	400	200	40	—	—

Table III—Dimethyl Amino-Styryl Compounds.

No.	Substance.	*Staphylococcus Aureus.*						*B. Coli.*						Precipitation.	
		Peptone water.			Serum.			Peptone water.			Serum.			P.	S.
		+	±	−	+	±	−	+	±	−	+	±	−		
13	$p\text{-}(CH_3)_2N.\overset{2}{St}.P.$ (Me I)	100	—	40	100	—	40	20	10	4	100	40	20	—	—
14	$p\text{-}(CH_3)_2N.\overset{2}{St}.Q$ (Me Cl)	200	—	100	200	—	100	20	—	10	100	—	40	—	—
15	$p\text{-}(CH_3)_2N.\overset{2}{St}.Q$ (Me I)	1000	400	200	1000	—	400	100	—	40	400	200	40	—	—
16	$p\text{-}(CH_3)_2N.\overset{2}{St}.Q.\overset{6}{CH_3}$ (Me Cl)	2000	—	1000	2000	1000	400	200	—	100	400	200	100	—	—
17	$p\text{-}(CH_3)_2N.\overset{2}{St}.\beta$ naphtho Q. (Me Cl)	1000	—	400	400	—	200	200	100	20	100	40	20	10	—
18	$p\text{-}(CH_3)_2N.\overset{2}{St}.Q.\overset{6}{OCH_3}$ (Me I)	2000	400	200	1000	—	400	200	100	40	200	—	100	—	—
19	$p\text{-}(CH_3)_2N.\overset{2}{St}.Q.\overset{6}{OC_2H_5}$ (Me I)	4000	2000	1000	2000	1000	400	400	100	20	1000	400	100	—	—
20	$p\text{-}(CH_3)_2N.\overset{2}{St}.Q.\overset{6.7.}{\genfrac{}{}{0pt}{}{O}{O}}\!\!>\!CH_2$ (Me S)	200	100	20	200	—	100	40	4	?	200	—	100	—	—
21	$p\text{-}(CH_3)_2N.\overset{2}{St}.Q.\overset{6}{NH_2}$ (Me Cl)	400	200	100	400	200	100	200	100	20	400	200	40	—	—
22	$p\text{-}(CH_3)_2N.\overset{2}{St}.Q.\overbrace{NH.COH}^{6}$ (Me Cl)	100	40	?	200	100	40	1	—	?	40	20	1	40	—

23	$p\text{-}(CH_3)_2N.St\overset{2}{.}Q.\overbrace{NH.COCH_3}^{6}(HCl)$	10	4	?	4	—	?	1	—	?	1	—	?	10	10
24	$p\text{-}(CH_3)_2N.St\overset{2}{.}Q.\overbrace{NH.COCH_3}^{6}(MeCl)$	400	200	40	200	100	40	1	—	?	100	40	20	20	—
25	$p\text{-}(CH_3)_2N.St\overset{2}{.}Q.\overbrace{NH.COCH_3}^{6}(MeS)$	100	40	?	100	40	20	2	—	?	100	—	40	10	—
26	$p\text{-}(CH_3)_2N.St\overset{2}{.}Q.\overbrace{NH.COCH_3}^{6}(EtS)$	100	40	10	100	40	20	1	—	?	40	20	10	10	—
27	$p\text{-}(CH_3)_2N.St\overset{2}{.}Q.\overbrace{NH.COC_2H_5}^{6}(MeCl)$	1000	400	200	400	—	200	20	10	?	400	200	40	20	—
28	$p\text{-}(CH_3)_2N.St\overset{2}{.}Q.\overbrace{NH.COC_3H_7}^{6}(MeCl)$	1000	400	200	400	—	200	100	40	4	400	200	100	4	—
29	$p\text{-}(CH_3)_2N.St\overset{2}{.}Q.\overbrace{NH.CO.C_4H_9}^{6}(MeCl)$	1000	—	400	400	200	100	40	20	10	400	200	100	10	1
30	$p\text{-}(CH_3)_2N.St\overset{2}{.}Q.\overbrace{NH.CO.C_5H_{11}}^{6}(MeCl)$	?	1000	200	1000	—	400	20	—	10	200	—	100	10	—
31	$p\text{-}(CH_3)_2N.St\overset{2}{.}Q.\overbrace{NH.CO.CH(C_2H_5)_2}^{6}$ (Me Cl)	1000	400	100	400	200	100	1	—	?	100	—	40	10	—
32	$p\text{-}(CH_3)_2N.St\overset{2}{.}Q.\overbrace{NH.CO.CBr(C_2H_5)_2}^{6}$ (Me Cl)	4000	—	1000	1000	400	200	100	40	2	100	—	40	10	4

Table III—(continued).

No.	Substance.	*Staphylococcus Aureus.*						*B. Coli.*						Precipitation.	
		Peptone water.			Serum.			Peptone water.			Serum.			P.	S.
		+	±	—	+	±	—	+	±	—	+	±	—		
33	$p\text{-}(CH_3)_2N.\overset{2}{St}.Q.\overbrace{NH.CO.CH_2Cl}^{6}$ (Me Cl)	400	200	10	40	20	10	2	—	?	40	—	20	10	4
34	$p\text{-}(CH_3)_2N.\overset{2}{St}.Q.\overbrace{NH.CO.CH_2Br}^{6}$ (Me Cl)	400	200	8	40	20	10	2	—	?	40	20	2	8	—
35	$p\text{-}(CH_3)_2N.\overset{2}{St}.Q.\overbrace{NH.CO.CCl_3}^{6}$ (Me Cl)	400	200	40	200	100	40	1	—	?	100	40	20	10	—
36	$p\text{-}(CH_3)_2N.\overset{2}{St}.Q.\overbrace{NH.COCH_3}^{7}$ (Me S)	100	40	2	200	100	40	1	—	?	100	40	20	10	2
37	$p\text{-}(CH_3)_2N.\overset{2}{St}.Q.\overset{6}{N}(CH_3)_2$ (Me Cl)	2000	1000	400	400	200	100	1000	—	400	400	—	200	—	—
38	$p\text{-}(CH_3)_2N.\overset{2}{St}.Q.\overbrace{N=N(H\text{-}Acid)}^{6}$ (Me Cl)	4	2	?	10	4	?	1	—	?	1	—	?	20	—
39	$p\text{-}(CH_3)_2N.\overset{2}{St}.Q.\overbrace{NH.COCH_3}^{6}$ (Me Cl) sulphonated	1000	400	100	1000	400	200	400	200	40	400	200	100	—	—
40	$p\text{-}(CH_3)_2N.\overset{4}{St}.Q.\overset{6}{C}H_3$ (Me Cl)	400	200	40	200	100	40	40	—	20	40	—	20	—	—

Table IV.—Amino-Anil Compounds.

No.	Substance.	*Staphylococcus Aureus.*						*B. Coli.*						Precipitation.	
		Peptone water.			Serum.			Peptone Water.			Serum.			P.	S.
		+	±	−	+	±	−	+	±	−	+	±	−		
41	$p\text{-}NH_2\overset{2}{A}.Q.(Me\,Cl)$	100	40	20	40	—	20	40	20	4	100	40	10	—	—
42	$p\text{-}NH_2\overset{2}{A}.Q.\overset{6}{CH_3}(Me\,Cl)$	200	100	40	200	100	40	100	40	20	100	—	40	—	—
43	$p\text{-}NH_2\overset{2}{A}.Q.\overset{6}{NH_2}(Me\,Cl)$	40	20	10	40	20	10	40	20	4	40	20	4	—	—
44	$p\text{-}NH_2\overset{2}{A}.Q.\overset{7}{NH_2}(Me\,Cl)$	100	—	40	100	—	40	20	10	4	40	20	10	—	—
45	$p\text{-}NH_2\overset{2}{A}.Q.\overbrace{NH.COCH_3}^{6}(Me\,Cl)$	40	20	10	40	—	20	40	20	10	200	100	40	—	—
46	$p\text{-}NH_2\overset{2}{A}.Q.\overset{6}{N}(CH_3)_2(Me\,Cl)$	100	—	40	200	—	100	40	20	4	200	100	40	—	—

Table V.—Dimethyl and Diethyl Amino-Anil-Compounds.

No.	Substance.	*Staphylococcus Aureus.*						*B. Coli.*						Precipitation.	
		Peptone water.			Serum.			Peptone water.			Serum.			P.	S.
		+	±	−	+	±	−	+	±	−	+	±	−		
47	p-$(CH_3)_2N . \overset{2}{A} . Q . (Me\,Cl)$	400	200	100	400	200	100	400	200	100	?	1000	200	—	—
48	p-$(CH_3)_2N . \overset{2}{A} . Q . \overset{6}{CH_3}\,(Me\,Cl)$	1000	400	200	1000	400	200	1000	400	200	1000	400	200	—	—
49	p-$(C_2H_5)_2N . \overset{2}{A} . Q . \overset{6}{CH_3}\,(Me\,Cl)$	1000	—	400	1000	400	100	400	—	200	200	—	100	—	—
50	p-$(C_2H_5)_2N . \overset{2}{A} . Q . \overset{6}{CH_3}\,(Me\,I)$	1000	400	100	400	200	100	100	—	40	200	100	40	—	—
51	p-$(CH_3)_2N . \overset{2}{A} . Q . \overset{6}{CH_3}\,\overset{8}{CH_3}\,(Me\,Cl)$	400	—	200	100	40	20	400	—	200	200	—	100	—	—
52	p-$(CH_3)_2N . \overset{2}{A} . \beta$ naphtho $Q . (Me\,Cl)$	?	4000	2000	2000	1000	400	?	2000	400	?	2000	400	—	—
53	p-$(CH_3)_2N . \overset{2}{A} . Q . \overset{6}{OCH_3}\,(Me\,Cl)$	2000	1000	200	200	—	100	200	100	40	1000	400	100	—	—
54	p-$(CH_3)_2N . \overset{2}{A} . Q . \overset{6}{OC_2H_5}\,(Me\,Cl)$	1000	—	400	1000	—	400	400	—	200	2000	1000	200	—	—
55	p-$(CH_3)_2N . \overset{2}{A} . Q . \overset{6\,.\,7}{\begin{smallmatrix}O\\O\end{smallmatrix}}\!>\!CH_2\,(Me\,S)$	100	40	20	200	—	100	40	20	10	400	200	100	2	—

56	$p\text{-}(CH_3)_2N . \overset{2}{A} . Q . \overset{6}{NH_2}$ (Me Cl)	400	—	200	400	200	100	200	—	100	400	200	100	—	—
57	$p\text{-}(CH_3)_2N . \overset{2}{A} . Q . \overset{6}{NH_2}$ (Me I)	40	20	10	40	20	10	20	—	10	100	40	10	—	—
58	$p\text{-}(CH_3)_2N . \overset{2}{A} . Q . \overbrace{NH . COH}^{6}$ (Me Cl)	100	40	20	100	—	40	100	—	40	400	200	100	—	—
59	$p\text{-}(CH_3)_2N . \overset{2}{A} . Q . \overbrace{NH . COCH_3}^{6}$ (Me Cl)	2000	1000	400	400	—	200	1000	—	400	2000	1000	400	—	—
60	$p\text{-}(CH_3)_2N . \overset{2}{A} . Q . \overbrace{NH . COCH_3}^{6}$ (Me Br)	?	1000	200	400	200	100	1000	—	400	?	1000	400	—	—
61	$p\text{-}(CH_3)_2N . \overset{2}{A} . Q . \overbrace{NH . COCH_3}^{6}$ (Me I)	1000	400	200	1000	400	200	1000	400	40	?	—	1000	—	—
62	$p\text{-}(CH_3)_2N . \overset{2}{A} . Q . \overbrace{NH\ COCH_3}^{6}$ (Me S)	1000	—	400	400	200	40	400	—	200	?	1000	400	—	—
63	$p\text{-}(CH_3)_2N . \overset{2}{A} . Q . \overbrace{NH . COCH_3}^{6}$ (Et S)	?	1000	200	1000	400	200	200	100	40	1000	400	200	—	—
64	$p\text{-}(C_2H_5)_2N . \overset{2}{A} . Q . \overbrace{NH . COCH_3}^{6}$ (Me Cl)	?	1000	400	1000	400	200	400	200	100	1000	400	200	—	—
65	$p\text{-}(CH_3)_2N . \overset{2}{A} . Q . \overbrace{NH . COC_2H_5}^{6}$ (Me C	?	?	1000	1000	400	200	?	1000	200	?	?	1000	—	—
66	$p\text{-}(CH_3)_2N . \overset{2}{A} . Q . \overbrace{NH . COC_3H_7}^{6}$ (Me Cl)	1000	400	200	200	100	40	400	—	200	?	4000	400	—	—

Table V—(continued).

No.	Substance.	*Staphylococcus Aureus.*						*B. Coli.*						Precipitation.	
		Peptone water.			Serum.			Peptone water.			Serum.			P.	S.
		+	±	−	+	±	−	+	±	−	+	±	−		
67	$p\text{-}(CH_3)_2N . \overset{2}{A} . Q . \overbrace{NH . COC_4H_9}^{6}$ (Me Cl)	1000	—	400	1000	400	200	4000	1000	400	2000	1000	400	—	—
68	$p\text{-}(CH_3)_2N . \overset{2}{A} . Q . \overbrace{NH . COC_5H_{11}}^{6}$ (Me Cl)	10000	4000	1000	1000	400	200	4000	2000	1000	4000	2000	400	10	—
69	$p\text{-}(CH_3)_2N . \overset{2}{A} . Q . \overbrace{NH . COCH}^{6} (C_2H_5)_2$ (Me Cl)	?	1000	400	?	1000	200	4000	2000	400	2000	1000	400	—	—
70	$p\text{-}(CH_3)_2N . \overset{2}{A} . Q . \overbrace{NH . COCBr (C_2H_5)_2}^{6}$ (Me Cl)	?	?	1000	400	200	100	1000	400	100	1000	400	40	—	—
71	$p\text{-}(CH_3)_2N . \overset{2}{A} . Q . \overbrace{NH . COCH_2Cl}^{6}$ (Me Cl)	1000	400	200	200	100	40	1000	400	200	400	200	100	—	—
72	$p\text{-}(CH_3)_2N . \overset{2}{A} . Q . \overbrace{NH . COCH_2 Br}^{6}$ (Me Cl)	400	—	200	100	40	10	100	—	40	400	200	100	—	—

73	$p\text{-}(CH_3)_2N\,.\,\overset{2}{A}\,.\,Q\,.\,\overbrace{NH\,.\,COC_6H_5}^{6}$ (Me A)	200	—	100	40	20	4	400	—	200	400	200	100	—	—
74	$p\text{-}(CH_3)_2N\,.\,\overset{2}{A}\,.\,Q\,.\,\overbrace{NH\,.\,COCH_3}^{7}$ (Me Cl)	2000	1000	400	2000	1000	400	400	200	100	1000	400	200	—	—
75	$p\text{-}(CH_3)_2N\,.\,\overset{2}{A}\,.\,Q\,.\,\overset{6}{N}(CH_3)_2$ (Me Cl)	1000	400	200	400	200	100	100	—	40	400	—	200	—	—
76	$p\text{-}(CH_3)_2N\,.\,\overset{2}{A}\,.\,Q\,.\,\overset{6}{N}(CH_3)_2$ (Me I)	1000	—	400	400	200	100	200	—	100	200	—	100	—	—
77	$p\text{-}(CH_3)_2N\,.\,\overset{2}{A}\,.\,Q\,.\,\overset{6}{Cl}$ (Me Cl)	400	200	100	200	—	100	200	—	100	200	—	100	—	—
78	$p\text{-}(CH_3)_2N\,.\,\overset{2}{A}\,.\,Q\,.\,\overset{6}{Br}$ (Me Cl)	200	100	40	20	—	10	40	20	10	40	20	4	—	—
79	$p\text{-}(CH_3)_2N\,.\,\overset{2}{A}\,.\,Q\,.\,\overset{6}{NO_2}$ (Me Cl)	100	—	40	20	10	4	200	100	4	10	—	4	10	4
80	$p\text{-}(CH_3)_2N\,.\,\overset{2}{A}\,.\,Q\,.\,\overbrace{N = N\,(H\text{-}Acid)}^{6}$ (Me Cl)	1	—	?	1	—	?	1	—	?	1	—	?	200	—
81	$p\text{-}(CH_3)_2N\,.\,\overset{2}{A}\,.\,Q\,.\,\overbrace{NH\,.\,COCH_3}^{6}$ (Me Cl) sulphonated	1000	400	200	1000	400	200	400	200	100	?	1000	200	—	—
82	$p\text{-}(CH_3)_2N\,.\,\overset{4}{A}\,.\,Q\,.\,\overset{6}{CH_3}$ (Me Cl)	100	—	40	100	40	20	10	—	4	10	—	4	—	—

Table VI.—Styryl and Anil Compounds with No Basic Group in the Benzene Ring.

No.	Substance.	*Staphylococcus Aureus.*						*B. Coli.*						Precipitation.	
		Peptone water.			Serum.			Peptone water.			Serum.			P.	S.
		+	±	–	+	±	–	+	±	–	+	±	–		
83	$\overset{2}{St} . Q . \overset{6}{CH_3}$ (Me Cl)	200	100	40	20	10	4	2	—	1	10	—	4	4	—
84	$\overset{m.}{OH} . \overset{p.}{OH} . \overset{2}{St} . Q$. (Me S)	20	4	2	4	2	1	1	?	?	2	—	1	10	2
85	$\begin{matrix} m.O \\ p.O \end{matrix}\rangle CH_2 \overset{2}{St} . Q$. (Me S)	40	20	10	10	4	2	1	—	?	20	—	10	1	—
86	$p\text{-}CH_3 CO . HN \overset{2}{St} . Q$. (Me Cl)	20	10	4	20	—	10	1	—	?	20	—	10	1	—
87	$p\text{-}CH_3 CO . HN . \overset{2}{St} . Q . \overset{6}{NH_2}$ (Me Cl)	2	—	?	20	10	4	1	—	?	1	—	?	100	4
88	$p\text{-}CH_3CO . HN . \overset{2}{St} . Q . \overbrace{NH . COCH_3}^{6}$ (Me Cl)	2	—	?	2	—	?	2	—	?	2	—	?	100	10
89	$p\text{-}CH_3CO . HN . \overset{2}{St} . Q . \overbrace{NH . COCH_3}^{6}$ (Me S)	1	—	?	1	—	?	1	—	?	1	—	?	100	20

90	$p\text{-CH}_3\text{CO . HN . }\overset{2}{\text{St}}\text{ . Q . }\overset{6}{\text{N}}\text{(CH}_3)_2\text{ (Me Cl)}$	40	20	1	100	—	40	1	—	?	10	—	4	—	—
91	$\overset{2}{\text{A}}\text{ . Q . }\overset{6}{\text{CH}_3}\text{ (Me Cl)}$	200	—	100	20	—	10	1	—	?	2	—	1	—	—
92	$\overset{2}{\text{A}}\text{ . Q . }\overbrace{\text{NH . COCH}_3}^{6}\text{ (Me Cl)}$	1	—	?	2	—	1	1	—	?	1	—	?	—	—
93	$\overset{2}{\text{A}}\text{ . Q . }\overset{6}{\text{N}}\text{ (CH}_3)_2\text{ . (Me Cl)}$	40	20	10	200	100	20	20	4	2	400	200	100	—	—
94	$p\text{-CH}_3\text{CO . HN . }\overset{2}{\text{A}}\text{ . Q . }\overbrace{\text{NH . COCH}_3}^{6}$ (Me Cl)	2	—	1	2	—	1	2	—	1	2	—	1	—	—

On the Effects of Beta Rays from Radium upon Division and Growth of Cancer Cells.

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(Communicated by Prof. A. W. Porter, F.R.S.—Received March 10, 1926.)

(From the Pathological Laboratories of the Radium Institute, London, and the Cancer Research Laboratories of the Middlesex Hospital.)

Introduction.

A dose of Beta rays can be given to Jensen's rat sarcoma (J.R.S.) which is just enough to prevent the cells growing when transplanted into normal susceptible rats; this has been referred to as the lethal dose (1). It is not known whether this effect is produced by inhibiting mitosis or whether growth is interfered with so that cells fail to mature, thus cutting off the supply of potentially dividing cells.

The following experiments were designed to show especially how the growth and division of sarcoma cells are affected by exposure to measured doses of Beta radiation. Independent experiments by Hertwig (2) and by one of us (3) have shown that cells are most susceptible to radiation when they are in a state of division. On the other hand, it was shown by Wedd (4) that cells of a mouse carcinoma which had received a "lethal dose" of Beta rays could still be found in a state of mitosis as long as six days after they had been inoculated into a susceptible animal. It will be seen, however, from the context, that it is possible to reconcile the idea of the state of mitosis being one which is peculiarly vulnerable to radiation with the fact that, after a lethal dose, numbers of the cells are afterwards found in mitotic activity.

Strangeways and Oakley (5) have studied in detail the action of doses of X-rays upon *in vitro* cultures of the choroid of chick embryos. In a general discussion of their results, they state that, although immediately after exposure to small doses of X-rays no effects upon division can be seen, yet if the cultures are returned to the incubator for 80 minutes and re-examined, marked inhibitory effects upon mitoses are evident. These authors also state that "if cultures in which mitosis has ceased (due to larger doses of X-rays) are returned to the incubator for some hours, cells in mitosis are again found, and unless a heavy dose of X-rays had been given, they become plentiful, but after the longer exposures the mitosis is abnormal."

Regaud, Lacassagne and Jovin exposed chickens' eggs to heavy doses of X-rays. They found numerous cells in mitosis at the end of the exposure, but none 15 minutes later. There was some return of mitotic figures afterwards, but many abnormal and degenerate cells were observed; after 3 days the embryo was dead. The experiments recorded here show that a rather close parallel exists between the sequence of changes after irradiation in the cells of the *in vitro* cultures, studied by Strangeways and Oakley, and that occurring in Jensen's rat sarcoma growing in a rat.

The Normal Variation of Mitosis in J.R.S.

Growing tumours of J.R.S. may vary widely in the percentage of cells in mitosis. Progressively growing tumours only were used in these experiments, and it was found that the most rapidly growing tumours did not necessarily give the highest mitotic count. The frequency of mitosis is here represented by the number of cells seen to be in division by a count of 50 fields. The conditions of magnification were lens 1/12-inch, eye-piece No. 4, tube length 150 mm. The size of field was 0·22 mm. diameter, and all stages of mitosis were included in the count.

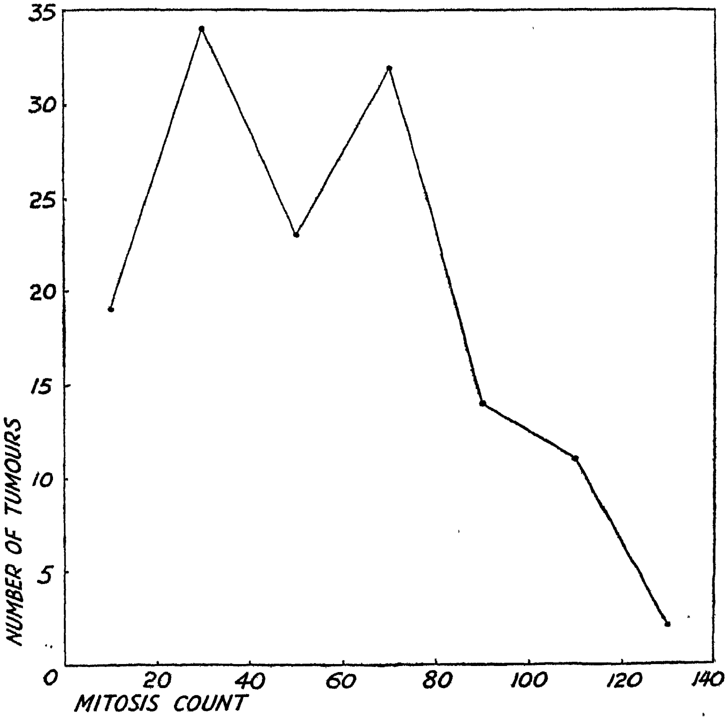

Fig. 1.

The distribution of the frequency of mitosis in normal tumours may be seen from the graph (fig. 1), which represents counts upon tumours in 135 different rats.

The reason for the wide extent of this variation is not apparent. If two tumours be grown in the same animal and then examined for mitoses, no greater range of variation than 2 : 1 occurs, as may be seen from the following cases:—

Table I.—Two tumours growing in the same rat.

Rat.	Number of mitoses in the two tumours.		Rat.	Number of mitoses in the two tumours.	
1	11	17	15	50	72
2	14	15	16	54	101
3	15	29	17	58	65
4	16	33	18	61	68
5	22	32	19	62	86
6	27	40	20	62	86
7	30	30	21	65	77
8	30	50	22	66	69
9	33	46	23	68	72
10	43	69	24	76	84
11	45	69	25	80	118
12	46	78	26	81	86
13	49	55	27	88	90
14	49	59	28	106	114

To see whether there was any connection between mitotic activity and the alternations of night and day, such as is known to be the case for the root tips of plants, two rats were inoculated in four different places; tumours grew, and they were taken out eight days later, at six-hour intervals, beginning at noon.

Table II.

Rat.	Number of mitoses in tumour removed at:—			
	Noon.	6 p.m.	Midnight.	6 a.m.
1	106	66	54	64
2	104	78	47	70

From the data given in Table II, it is seen that the highest counts occurred at midday.

In order to test this point more fully, 20 rats were inoculated in four different places; at the end of ten days, 16 rats were used. They were divided into two groups, A and B.

Group A (8 rats).—Two tumours were removed from each rat at midday and the other two at the next midnight.

Group B (8 rats).—Two tumours were removed at midnight and the other two on the following day at midday. The object of this grouping was to eliminate the effect of the operation upon the mitosis count.

The following data were obtained:—

Group A.

No.	Midday count.	Midnight count.
1	56	51
2	62	36
3	71	70
4	84	52
5	74	60
6	89	56
7	99	49
8	109	46
Average	80	52

Double the usual number of fields were counted for this experiment (*i.e.*, 100 instead of 50).

Group B.

No.	Midnight count.	Midday count.
1	41	61
2	54	62
3	62	67
4	66	54
5	68	70
6	68	80
7	70	61
8	70	74
Average	62	66

From the data it may be concluded that the variation in the frequency of mitosis at midday and midnight, if it occurs at all, does not exceed the variations which may occur among two tumours growing in the same animal and removed at the same time, and that this variation is certainly not enough to account for the wide range in the mitotic count shown in fig. 1. The data do, however, suggest that an operation tends to lower the number of mitoses in a tumour left in the animal.

The Effects of Radiation upon the Mitotic Count.

In order to see what effect the Beta rays have upon mitosis, rats were inoculated in the right and left axillæ with J.R.S. When small tumours measuring about 12 × 12 mm. had grown, a radium capsule* containing 30 mgms. of radium bromide was held to the surface of one of the tumours for a specified time, the animal being under an anæsthetic. When the exposure was over a piece of tumour less than 1 mm. thick immediately below the skin in the middle of the part irradiated was taken for microscopic examination, and a similar piece from the control tumour. These were put at once into 33 per cent. acetic alcohol; sections were made 1/200 mm. thick and faintly stained with iron hæmatoxylin, and the number of mitoses per 50 fields was counted under a 1/12 objective, with a No. 4 eye-piece, tube at 15·0.

Three series were carried out with exposures which represented one-half the lethal dose (½ L), the lethal dose (L) and twice the lethal dose (2 L). The actual lengths of time for the radium exposures were ½ hour, 1 hour, and 2 hours. The data for the three series are collected in fig. 2.

Inspection of the graph shows that, one hour after a dose ½ L, there are practically no cells in mitosis in the irradiated tumour, but if the irradiated tumour be left in the animal for 24 hours, there is no appreciable difference between the number of its mitoses and that of the control.

When the dose is increased to the full lethal dose (L) the same phenomenon occurs one hour after the exposure, *i.e.*, an almost complete absence of mitosis in the irradiated tumour; this condition now lasts much longer than before, but in about two days' time there is an appreciable return of cells to the stage of mitosis; after five days, however, there are very few to be seen. Counts, five or more days after a lethal dose, are complicated by the disappearance of sarcoma cells.

When the dose is increased to 2 L the same phenomenon occurs, and if the irradiated tumour cells are left in the animal for four days, there is a very considerable return of cells into mitosis, in spite of their having received a dose twice as large as is necessary to prevent their ever developing into a tumour.

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* The radium salt was contained in a circular silver box about 2 mm. deep and 1 cm. diameter fitted with a window of silver 0·1 mm. thick.

Some cells in mitosis are to be seen in the sixth-day sections. After six days, very few sarcoma cells are seen and none in mitosis.

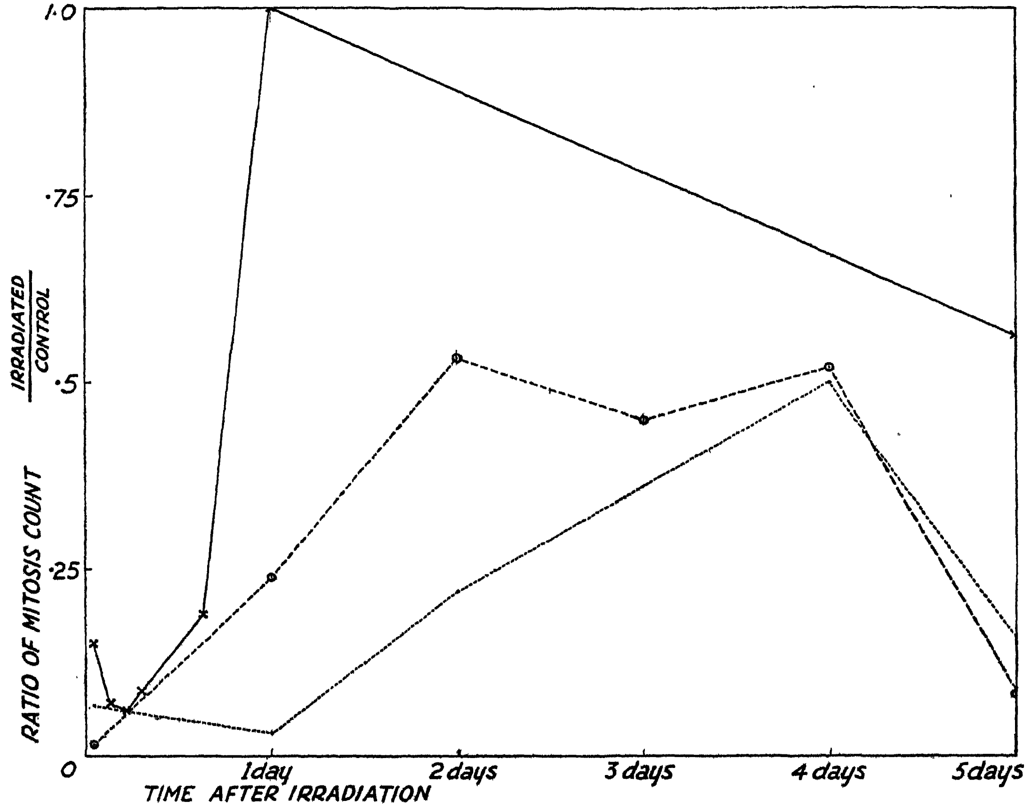

FIG. 2.

× ——— ½ hour exposure.
⊙ - - - - 1 hour ,,
● .... 2 hours' ,,

Cell activity in a tumour may, for our present purpose, be considered to have two phases of particular importance :—

1. The phase of growth of the immature cell to maturity, at the end of which it is ready to go into mitosis.
2. The phase when the cell is undergoing mitosis and subsequent cleavage whereby two daughter cells originate.

Experiment has shown that immediately after a lethal dose has been given to the tumour there are plenty of cells in mitosis, but that one hour later cells in mitosis can hardly be found, and that after this same dose the cells, if allowed to remain in the body, come into mitosis again; yet eventually they produce no tumour.

If we assume that cells undergo mitosis even after a lethal dose, but that the newly produced daughter cells are so profoundly affected that they never reach maturity and that growing cells (phase 1) are temporarily prevented from coming into mitosis, the effects of three such processes would tally with experimental results.

It has been found by one of us (7) that, for a normal progressive J.R.S., the time taken for the whole cycle of cell maturity and division is about four days, and that the phase of division lasts about one hour.

The experiments just referred to may, perhaps, be thought to support this deduction, because immediately after a lethal dose (L) which takes one hour, no effect upon mitosis is recognisable, but one hour later the effect is very marked. It will be noticed, too, that the return to mitosis, which occurs after a lethal dose or more, goes on for about five days, which is approximately the time required for maturity; at the end of this time, the only cells approaching maturity would be the daughter cells of the irradiated ones. These, by supposition, have been so influenced by the radiations that they do not go into mitosis.

The deduction of the time taken for division has, however, been tested in a different way. A container holding a large quantity of radium emanation (radon) was prepared, so that a lethal dose took only four minutes, possibly less. The container was a shallow box of exactly the same size as the radium capsule; it was fitted with a silver window and filled with 170 millicuries. Experiments were made in the way which has been described, and counts of the relative numbers of mitotic figures, in control and irradiated tumours growing in the same animal, were made at various intervals after the four minutes' exposure.

It will be seen from the data in Table III that, although hardly any mitotic figures are to be found one hour after this exposure, something like this interval is necessary before such a stage is reached.

Using such strong sources, it was found that the length of the exposure was not so long as would be estimated by the ordinary method of calculating by intensity × time. For instance, with 180 millicuries in a capsule of the same dimensions as that containing 30 milligrams of radium bromide, an exposure lasting 2·5 minutes should be necessary in order to produce the typical effect that is obtained one hour after a 30-minutes' exposure to the radium bromide; but actually this effect can be obtained by an exposure of 30 seconds.

Table III.

Radium emanation.	Length of exposure.	Length of time between end of exposure and removal of tumour.	Mitoses.	
			Irradiated.	Control.
Millicuries.	Minutes.	Hr. Min.		
170	4	0 2	45	99
170	4	0 2	102	80
170	4	0 10	54	72
170	4	0 20	22	27
170	4	0 40	21	22
170	4	0 40	2	77
170	4	0 50	1	115
170	4	1 0	3	106
170	4	1 0	4	44

It has been seen from the above experiments that, one hour after a 30 minutes' dose (*i.e.*, ½ L), very few cells in mitosis can be found; small pieces of tumour removed *immediately* after this appeared to have more than their normal number, so it was decided to trace what happened during the hour between the irradiation and the removal of the tumour from the animal. Twelve tumours were given the 30 minutes' dose and pieces removed from them immediately afterwards. Twelve more were given the same dose and removed 10, 20, 40 and 60 minutes afterwards. In each case control tumours were grown in the same animals and excised for comparative counts. In most cases two control tumours were grown, in order to get a fair average normal count. The data are given in Table IV and shown in fig. 3.

It will be seen that these data provide evidence that there is real though quite temporary increase in the number of cells in mitosis, just after one-half of the lethal dose has been given to a tumour. Previous observations (p. 328) have shown that two tumours growing in the same animal may, though rarely do, give counts varying as much as 2 : 1, but this ratio is exceeded in 5 out of the 12 cases examined immediately after the irradiation.

An increase in the number of mitoses found after irradiation need not necessarily be looked upon as a stimulation effect; if the act of division into two daughter cells were hindered, an increase in the numbers of cells seen in mitosis might result temporarily.

Table IV.—Variation in Mitosis in Tumour after 30 minutes' irradiation ($\frac{1}{2}$ L).

Time after exposure.	Mitoses count.	
	Control.	Irradiated.
Immediately	4 12 14 14 21 22 24 24 27 30 40 82	22 31 19 44 58 33 68 26 44 39 35 58
10 minutes	30 34	39 42
20 minutes	20 69	16 14
40 minutes	27 57	11 2
1 hour	17 42 43 49 63 91	6 3 31 2 1 4

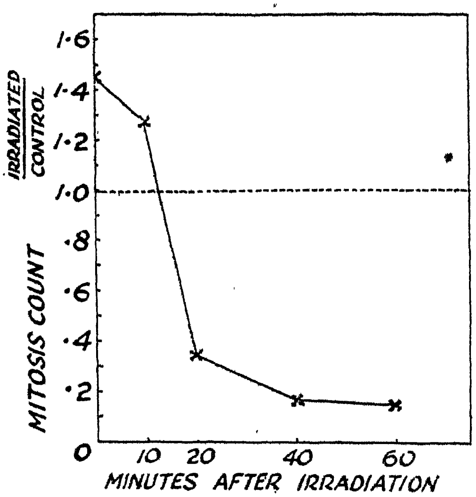

Fig. 3.

(Minutes after exposure.)

Conclusion.

Experiments are described which show that the action of the Beta rays of radium upon tumour cells is complicated by the various phases of cell activity which are represented by growth and division.

Evidence is brought to show that the cells of J.R.S. in a state of division suffer no *immediate* recognisable changes on exposure to large doses of Beta radiation.

The *subsequent* changes, involving an absence of cells in active division, are interpreted as being due to a temporary inhibition of maturing cells entering the phase of mitosis.

The *final* changes, which are profound enough to prevent growth of the tumour when inoculated, may be interpreted as being due to the fact that eventually after a lethal dose of radiation, the daughter cells are not capable of subsequent normal division.

We have enjoyed the facilities of strong sources of Radon through the courtesy of the Medical Research Council.

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The Excretion of Uric Acid by the Kidney.

By Hans Gremels and Richard Bodo (Rockefeller Travelling Fellows).

(Communicated by E. H. Starling, Foulerton Professor of the Royal Society. — Received June 16, 1926.)

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The excretion of uric acid by the kidney has played an important part in the investigations and speculations concerning the mechanism of urinary secretion. The microscopic investigation of the kidney, after the injection of uric acid or the administration of large amounts of purines, has shown the presence of granules of uric acid in the cells of the convoluted tubules and a precipitate of this substance in the lumen of the tubules (Heidenhain, Nussbaum, Ebstein, Nikolaier, Sauer, Minkowski, Eckert and others) (1). These appearances have been held to support Heidenhain's theory that the convoluted tubules are the seat of secretion of uric acid.

Other observers, and especially Cushny (2) in his presentation of the modern theory of secretion, do not accept this interpretation, but regard the granules as due to a process of absorption of the substance from the dilute filtrate formed by the glomeruli and passing down the tubules. According to Folin (3) the kidney of the dog is able to store considerable amounts of injected uric acid and to return it to the blood stream later. This observer described a very rapid disappearance of injected uric acid from the blood, which he ascribes to oxidation in the blood itself as it passes through the tissues. A more detailed discussion of Folin's results will be given later.

In view of the great difficulty of deciding between the alternative views as to the origin of the granules, by secretion or by absorption, it seemed that new light might be thrown on the problem by an investigation of the behaviour of the isolated perfused kidney towards uric acid, and it is the purpose of this paper to describe the experiments which have been made in this direction.

In the investigation by Starling and Verney (4) of secretion in the surviving dog's kidney, in which the circulation is maintained by means of a heart-lung preparation, these authors concluded that the process occurring in the glomeruli was one of simple filtration, the glomerular filtrate representing the plasma of the blood deprived of its proteins and any other colloid constituents. This filtrate, however, was not, as imagined by Cushny, 80 or 100 times the final amount of fully formed urine, but exceeded the latter little, if at all, in amount.

As this glomerular transudate passed down the tubules it lost in the perfused kidney nearly the whole of its chlorides, unless pituitrin were present in the circulating blood. It might lose also a certain amount of water, but in most cases not more than 50 per cent. of its bulk, and in some cases none at all. To this transudate were added on its way down the tubule certain products of secretion by the convoluted tubules, namely, urea and, when these substances were injected into the blood stream, sulphate and phenol red. It has also been shown (Brull, Eichholtz, and Robison (5)) that if organic phosphates are present in the blood, these are taken up into the kidney cells (presumably the cells of the convoluted tubules) in a concentrated form, undergo hydrolysis in whole or in part, and are excreted in concentrated form as inorganic phosphates into the glomerular transudate on its way down the tubule. The evidence for secretion is strongest in the case of the phenol red and of the phosphate. We hoped that similar methods might throw light on the manner of excretion of uric acid.

Methods.—All our experiments have been carried out on dogs, using the heart-lung-kidney preparation as described by Starling and Verney. In certain experiments we devised small modifications which will be described later. It is important, for the success of these experiments, to bear in mind the poisonous effect on the kidney and its blood vessels of the defibrinated blood necessarily used in the heart-lung preparation. As Eichholtz and Verney (6) found, defibrinated blood causes a strong contraction of the kidney vessels supplying the flow of blood through the organ and thus prevents any flow of urine. As the blood becomes detoxicated through circulation through the heart-lung preparation, the blood flow and urine flow gradually increase. It is therefore important to wait some time, after completing the heart-lung preparation, before connecting up the kidney, and if this precaution is observed, the urine flow may commence directly the circulation is started through the excised kidney. In none of our experiments did we have to wait more than 15 minutes for the inception of the urinary flow. This toxic effect seems to be absent when hirudinized blood is used instead of defibrinated blood, as we were able to do in a few cases. If a good urinary flow is desired, it is also desirable to introduce a fair amount of normal saline into the dog from which the kidney is taken. In Starling and Verney's technique the kidney dog, after being bled about 350 c.c., receives 350 c.c. of normal saline. This procedure has a diuretic effect during the following half-hour which elapses before the kidney is removed from the animal, and the diuresis seems to continue when the kidney is connected up to the heart-lung preparation, and seems to be still greater if the kidney dog has received an excess of normal saline, say, 400 to 450 c.c.

In all our experiments we determined the uric acid content of the blood and urine, the chlorides in the urine and sometimes in the blood, and in one group of experiments also the allantoin content of the urine. Chlorides were estimated by the Volhard method. For addition to the blood or injection into the animal we used the lithium salt of uric acid: this was prepared in the following way: 0·5 gm. of lithium carbonate was dissolved in 100 c.c. distilled water. This takes about half an hour. The solution was warmed on the water-bath and 1·0 gm. of uric acid dissolved in it. This again took half to three quarters of an hour. The solution should be stirred frequently. 4 gms. of dextrose were then added to the solution in order to make it isotonic with the blood. This precaution is extremely important, as otherwise the lithium urate solution produces hæmolysis and may have an injurious effect on the kidney. The warm solution was then made up to 100 c.c. to replace any fluid that had evaporated, and was used while still warm. If it be allowed to cool, a gelatinous precipitate may result. For the determination of uric acid a modification of Folin's method was employed, using the reagent as recommended by Folin and Trimble (7). For the determination of the uric acid content of both the blood and urine we used Folin's blood standard, and we diluted the test solution until the uric acid concentration became approximately the same as that of the standard, so that the colorimeter was used only as a null instrument. In the precipitation of the blood proteins 5 c.c. of blood were added to 84 c.c. of water with 5 c.c. of 10 per cent. sodium tungstate solution, 5 c.c. 2/3 normal H_2SO_4, and 1 c.c. 10 per cent. H_2SO_4. A clear, freshly made solution of sodium tungstate must always be used, and the acid must be added immediately after the sodium tungstate, otherwise the filtrate is not clear. This method of precipitating the proteins was also employed for the urine when this contained blood. To determine the accuracy of this method we compared the results obtained by Folin's and the Hopkins-Folin-Wu methods and also made a nitrogen determination by Kjeldahl's method. The following results were obtained :—

Theoretical value : 100·0 mgms.

1. Folin's uric acid method : 100.
2. Hopkins-Folin-Wu method : 94·6.
3. Kjeldahl's nitrogen determination : 99·89.

On adding the same amount of lithium urate solution to blood and precipitating all the proteins as already described, the determination gave only 70 to 75 per cent. of the original amount. This loss was constant, and a corresponding allowance must therefore be made in the figures given later in our protocols for the uric acid blood content.

Allantoin was determined by Wiechowski's method (8). The urine was precipitated with phosphotungstic acid in an acid medium and filtered through kieselguhr. The acids are neutralised with lead oxide and the chlorides of the clear filtrate precipitated with silver lactate. The lead and silver are then precipitated with hydrogen sulphide, the excess of the latter being driven off with a stream of air. The clear filtrate is then made alkaline with magnesium oxide, and finally allantoin is precipitated with 0·5 per cent. mercuric acetate solution. The urine must be diluted three or four times to avoid the precipitation of mercuric urea. The precipitate is then dissolved in 10 per cent. pure nitric acid and the mercuric allantoin titrated with sodium thio-cyanate. Control estimations by this method gave satisfactory results.

The Excretion of Uric Acid in the Heart-Lung-Kidney Preparation.—After the excision of the kidney and its connection with a heart-lung preparation with a blood pressure varying from 100 to 110 mm. Hg., the urinary flow started either immediately or within a few minutes. The urine was collected in a graduated vessel and the blood flow was measured in the same way by observing the time required to collect 100 c.c. of the outflowing blood. The blood flow and the urine flow gradually increased during the first 15 minutes to an optimum level, where they remain constant so long as the conditions of the experiment are the same. Towards the end of an experiment the urine flow may exhibit a decrease, the blood flow remaining constant. Time was always allowed for the blood and urine flow to reach a constant level before starting observations. 8 to 15 c.c. of urine were then collected and one blood sample taken. A given amount of uric acid, varying from 30 to 200 mgms., was then added to the blood in the venous reservoir. Nine experiments of this type were carried out; details of two of these may now be given.

In the experiment given in Table I the effect of adding 80 mgm. of uric acid to the 800 c.c. of blood in the reservoir and circulating through the heart-lung-kidney was to raise the uric acid content of the blood from 0 to 5 mgm. per cent. The remainder of the uric acid had been taken up mainly by the kidney, but also by the tissues of the heart-lung preparation. (Normal dog's blood does not show any appreciable trace of uric acid by the method used.) This rise in the uric acid of the blood was accompanied by a rise both in the volume of the urine and the uric acid content of the urine. The urine increased from 0·8 c.c. per minute to 1·3 c.c. per minute, at which level it remained constant for half an hour and then dropped to 1 c.c. per minute. During the last twenty minutes of the experiment the urinary flow fell to 0·014 c.c. per minute. The uric acid of the urine rose from 0·7 mg. per cent. to 20·3 mg. per cent., and at the end of the experiment to 33·3 mg. per cent. The chlorides of the urine,

Table I.—16.10.25. Heart-Lung-Kidney.

Time. mins.	Art. Pr. mm. Hg.	Blood flow. Kidney, cubic centimetre per min.	Urine flow, cubic centimetre per min.	Uric Acid.			NaCl. g. per cent.
				Blood mg. per cent.	Urine mg. per cent.	Urine abs. amount mg.	
1.31	110	103	0·8	0	8·7		0·013
1.42	80 mg. Uric Acid added.						
1.45	110	100	1·3	5·0	20·3	0·81	0·018
1.51	110	100	1·3		20·3	0·81	0·018
1.54	110	100	1·3	4·3*a* 4·8*v*	23·5	0·94	0·018
1.57	110	100	1·3		23·0	0·92	0·015
2.03	110	100	0·8		25·0	1·1	0·014
2.12	110	100	1·0	4·0	26·0	1·14	0·019
2.18	110	100	0·7		27·3	1·19	0·025
2.26	110	96·7	0·7		33·3	1·33	0·023
2.55	110	91·0	0·014	3·3*a* 3·5*v*	33·3	1·33	0·024

The urine flow started at 12.48. Until 1.30, 16 c.c. of urine had been secreted. In samples 2 and 4 separate estimations of uric acid have been made, *a* being the blood of the renal artery, *v* that of the renal vein.

as is invariably the case in the surviving kidney, were very low, so that the urine was markedly hypotonic. There was a slight increase in the chlorides after the administration of the uric acid, and this became more marked as the urine flow became slower at the end of the experiment.

If we compare the amount of uric acid disappearing from the blood with that excreted in the urine, we find that whereas 10·5 mg. disappeared from the blood, 9·7 mg. were excreted by the kidneys. After the injection of uric acid 11·6 per cent. was excreted, but apart from the initial disappearance of uric acid due to its being taken up by the tissues of the preparation the whole of the disappearance from the blood can be accounted for by the amount excreted. No appreciable difference was found between the uric acid of the arterial blood and of that flowing from the kidney. Two estimations gave 4·3 mg. per cent. arterial, 4·8 mg. venous ; 3·3 mg. arterial and 3·5 mg. per cent. venous. These differences are well within the limits of experimental error.

The results of another experiment are given in Table II. In this case uric acid was given in two doses, first a small dose of 30 mg. and then a large dose of 250 mg. The first amount raised the uric acid level in the blood to 2·7 mg. per cent., which dropped to 1·9 mg. per cent. in the course of an hour. On adding the larger dose the level rose to 11·4 mg. per cent., dropping to 7·5 mg.

Table II.—8.12.25. Heart-Lung-Kidney.

Time. mins.	Art. Pr. mm. Hg.	Blood flow, Kidney, cubic centimetre per min.	Urine flow, cubic centimetre per min.	Uric Acid.			NaCl. g. per cent.	Allantoin g. per cent.
				Blood mg. per cent.	Urine mg. per cent.	Urine abs. amount mg.		
1.14	110	120	0·8	0	3·3	0·33	0·017	0·235
1.25	30 mg. Uric Acid added to the venous reservoir							
1.35	30	125	1·2	2·7	6·30	0·63	0·028	
1.47	30	130	1·1		7·5	0·75	0·026	
2.04	30	130	1·2		6·7	1·34	0·026	
2.23	30	130	1·6	1·9	5·0	1·05	0·031	0·080
2.24	250 mg. Uric Acid added.							
2.29	110	130	2·2	11·4	21·5	2·37	0·036	
2.43	110	150	2·0		17·7	5·30	0·038	
2.55	110	150	3·3	8·3	12·9	4·37	0·047	
3.12	110	150	2·9		18·8	9·38	0·054	
3.24	110	150	1·7	7·5	14·3	4·29	0·059	0·053

12.23 the urine flow started. By 1.14 30 c.c. of urine had been excreted, which were used for the first allantoin estimation.

per cent. during the next hour. Associated with these changes the uric acid level in the urine rose first from 3·5 to 7·5 mg. per cent., and then to 21·5 mg. per cent., so there can be no doubt that the concentration of uric acid in the urine varies with the concentration of this substance in the blood. In this experiment the diuretic effect of the uric acid was much more pronounced than in the first, so that the enhanced urinary flow is responsible for the fact that the rate of fall of the blood uric acid in the second experiment was much more rapid than in the first. The highest percentage of uric acid in the urine was less than that obtained in the first experiment—namely, 21·5 mgm. as against 33·5 mgm.

The chlorides, as in the first experiment, showed a slight increase after the addition of the uric acid. In this experiment the allantoin of the urine was also estimated before and after the addition of uric acid. The uric acid caused no increase in the allantoin, but, on the contrary, the concentration of this substance diminished from 0·235 to 0·053 per cent; this result will be discussed later. As regards the absolute amounts of uric acid excreted during the first period, 4·9 mg. disappeared from the blood and 3·8 mg. were excreted. In the second period 27 mg. disappeared and 25·7 were excreted. Here again there is no evidence of any oxidation or destruction of the uric acid in the blood. These experiments show that the isolated kidney fed with defibrinated blood at a pressure of 100 to 110 mm. by a heart-lung preparation can excrete uric acid added to the blood in a greater concentration than that at which it exists in the latter fluid, the percentage of uric acid in the urine being from 3 to 12 times that of the blood. Moreover, after the addition of uric acid to the blood, an equilibrium is quickly established between the tissues and the blood, and after this initial diminution of uric acid all the uric acid which disappears can be accounted for by the excretion of this substance through the kidney. We have still to decide whether the concentration of uric acid by the kidney is due to actual secretion by the tubular cells or to a concentration by absorption of water from the dilute uric acid solution filtered through the glomeruli. To decide this question we carried out two further sets of experiments.

(1) *Low-Pressure Experiments on the Isolated Kidney.*—As already mentioned, Folin found a considerable accumulation of uric acid in the kidney when this substance was injected into the blood stream of a dog. This result by itself is not evidence of a selective absorption of this substance and its concentration within secreting cells of the tubules, since it might be due to the accumulation of uric acid in the lumen of all the tubules of the kidney below the convoluted

tubules. We attempted, therefore, to decide the question of the selective absorption of the uric acid of the blood by the renal cells by the same method as that employed by Starling and Verney in their experiments on the excretion of phenol red. It will be remembered that in the latter, after all possibility of glomerular filtration had been stopped by lowering the blood pressure in the renal artery to about 33 mm. Hg., the injection of phenol red gave rise to an accumulation of this substance in the convoluted tubules of the kidney, and the fact that the renal cells were taking it still from the blood was shown by a comparison of the dyestuff contained in the plasma of the arterial and venous blood respectively. In our experiments we stopped the urinary flow in the same way—namely, by lowering the blood pressure to approximately 35 mm. Hg. The results of such an experiment are given in Table III. After the collection of 22 c.c. of urine we dropped the perfusion pressure from 115 to 35 mm. Hg. The result was an instantaneous cessation of urine flow, though there was still a fair flow of blood through the organ, the blood flow per minute dropping only from 150 to 50 c.c. per minute. On now adding 200 mg. uric acid to the blood the uric acid concentration in the blood rose from 0 to 10·68 mg. per cent., at which level it remained constant throughout the experiment (80 minutes). We could detect no difference in the uric acid content of the arterial and venous blood.

Table III.—24.11.25. Heart-Lung-Kidney.

Time. mins.	Art. Pr. mm. Hg.	Blood flow, kidney, cubic centimetre per min.	Urine flow, cubic centimetre per min.	Uric Acid Blood mg. per cent.
1.17	115	150	0·62	
1.35	115	150	0·65	Trace
1.37	35	54·5	0	
1.53	35	50	0	200 mg. Uric Acid added.
1.57	35	50	0	10·68*a* 10·21*v*
2.03	35	50	0	10·00*a* 10·68*v*
2.1	35	50	0	10·00*a* 10·44*v*

The analysis of the perfused kidney at the end of the experiment showed that it contained 10 mg. per cent. uric acid, whereas the other kidney from the same dog, which had not been perfused, yielded only 4 mg. per cent. Similar results were obtained in five other experiments of the same type. The evidence for an accumulation of uric acid in the kidney in these circumstances is thus not so definite as in the case of the phenol red. The extraction of uric acid from the kidney where it exists as intracellular granules is, however, a much more difficult matter than the extraction of the alcohol-soluble phenol red. We attempted to improve the methods of extraction by grinding up the tissue after immersion in liquid air, but obtained the same results as when it was ground up with sand, as in Folin's method. The only situation where granules could be observed under the microscope was in the cells of the convoluted tubules. If the whole of the 10 mg. per cent. found in the kidney could be regarded as confined to these cells, we should have to assume a very considerable concentration of the uric acid of the blood by these cells. It is, however, not possible with our present methods of estimating uric acid in the tissues to be certain of this localisation of the whole of the deposit of uric acid.

(2) *Suspension of the Tubular Activity by Means of Cyanide.*—In order to decide whether uric acid is filtered and then concentrated by reabsorption of water or is actually secreted by the tubular cells, we adopted the device of Starling and Verney of paralysing the tubules with sodium cyanide. The method of these authors was followed, the only difference being that we used a cyanide concentration of M/200 instead of M/600. The latter concentration did not seem strong enough in our experiments for complete paralysis of the tubular functions of the kidney. In these experiments, after the urinary flow was established, the uric acid was added to the blood and the urine collected for from half to one hour. Perfusion with cyanided blood was then started and maintained for 15 minutes, care being taken to keep the mechanical conditions of the preparation, *e.g.*, blood pressure and blood flow, constant throughout. The kidney was then switched back to its original connection with the heart-lung preparation, care being taken to wash away all the cyanide from the kidney and apparatus before blood from the kidney was returned to the reservoir of the heart-lung apparatus. The results of a typical experiment are shown in Table IV and represented graphically in fig. 1.

After a short control period without uric acid, 160 mg. uric acid were added to the blood. This caused a rise in the uric acid content of the blood from zero to 8·6 mg. per cent. In the urine the uric acid rose from 4·36 to 52·2 mg. per cent. A quarter of an hour later the uric acid percentage in the urine was 80 mgms. Owing to the secretion of this substance the blood level diminished to 5·6 mg. per cent., while the uric acid in the urine fell to 23·5 mg. per cent. in the course of an hour. It was reckoned that 42 mg. of uric acid had disappeared from the circulating blood during this time, while the amount excreted

Table IV.—11.3.26. Heart-Lung-Kidney.

Time. mins.	Art. Pr mm. Hg.	Blood flow, Kidney, cubic centimetre per min.	Urine flow, cubic centimetre per min.	Uric Acid.			NaCl. Urine g. per cent.	NaCl. Serum g. per cent.
				Blood mg. per cent.	Urine mg. per cent.	Urine abs. am. mg.		
12.52	100	100	0·84	0	5·71		0·094	
12.58	100	115	0·94	0	4·36		0·094	0·432
12.59	Addition of 160 mg. Uric Acid.							
1.07	100	125	1·00		52·20	4·2	0·094	
1.10	100			8·60				
1.15	100	125	1·00		80·00	6·6	0·053	
1.25	100	125	1·00		60·00	6·0	0·053	
1.33	100	120	1·25		42·50	4·3	0·053	
1.39	100	120	1·67		38·71	3·9	0·074	
1.45	100	120	2·17		31·56	4·2	0·068	
1.50	100	120	2·00		27·36	2·7	0·086	
1.54	100	120	2·50		25·30	2·5	0·092	
2.00	100	120	2·50	5·60	23·53	2·2	0·092	0·440
2.00	Perfusion with M/200 Sodium-cyanide-Blood.							
2.06	120	150	3·10		7·50	0·8	0·352	
2.10	110	120	2·50		6·50	0·7	0·366	
2.14	110	120	2·50		5·71	0·5	0·432	
2.16	110	120	2·00	4·80	5·22	0·21	0·440	0·453
2.16	Again perfused by the Heart-Lung.							
2.28	100	68	2·20		6·00	0·7	0·298	
2.34	100	68	2·33		5·50	0·8	0·254	
2.40	100	71	1·66		5·00	0·5	0·146	
2.45	100	63	2·00		5·46	0·6	0·172	
2.51	100	50	0·66	3·80	8·00	0·3	0·210	

The preparation started at 12.21. Up to 12.52 12 c.c. of urine were secreted.

In the period before cyanide 46 mg. of uric acid had been secreted. From the 1400 c.c. of blood 42 mg. uric acid disappeared, being 38·2 per cent. of the total amount.

in the urine was 46 mgms., a very fair agreement. The urinary flow increased after the addition of the uric acid, first from 0·9 to 1 and later to 2·5 c.c. per minute, and the urinary chlorides were of the usual low order, varying during this time from 0·05 to 0·09 per cent. Perfusion of the kidney was now commenced with defibrinated blood withdrawn from the reservoir of the heart-lung apparatus to which M/200 hydrocyanic acid had been added. An endeavour was made to maintain the mechanical conditions of the perfusion as constant as possible. Apart from a temporary increase in blood flow, which rose from 120 to 150 c.c. for a few minutes owing to the employment of too high a pressure, this equality of conditions was achieved. The urinary flow rose to 3·1 c.c. per minute, to fall again to 2·5 c.c.—*i.e.*, the same volume as before—when the blood flow through the kidney was adjusted to be equal to the previous amount. The uric acid of the urine dropped immediately to 7·5 mg. per cent., and at the end of the period of perfusion it reached 5·22 mg. per cent. as against 4·8 mg. per

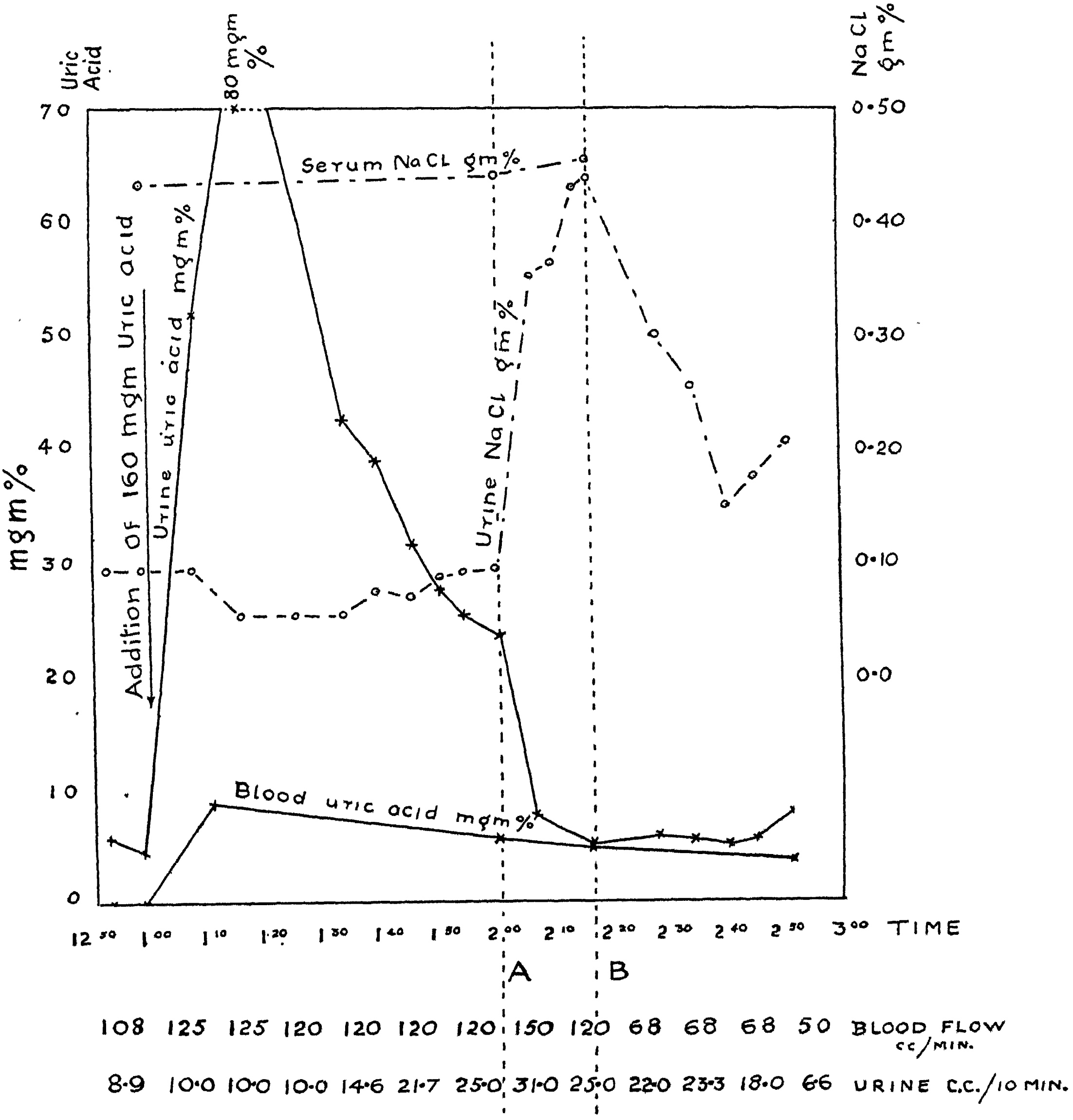

Fig. 1.—Graph of concentrations of uric acid and of sodium chloride in blood, blood serum and urine. Uric acid added to the blood at the arrow. Between A and B the kidney was perfused with cyanide blood.

cent. found in the blood plasma. Allowing for the lower figures always obtained in the titration of the uric acid of the blood serum in comparison with those of the urine, we may regard the uric acid content of the blood and urine as being practically identical at the end of the perfusion. Since the urinary flow remained nearly constant, the amount secreted per unit time was also diminished. At the same time the chlorides of the urine rose from 0·092 to 0·44 per cent., the blood serum value being 0·453 per cent.

We may therefore conclude that we have succeeded by the circulation of the cyanide blood in obtaining a pure glomerular filtrate. The kidney was now once more put into connection with the heart-lung preparation. The urinary flow remained nearly constant, although the blood flow through the kidney fell from 120 to about 70 c.c. per minute, the arterial pressure being maintained at the previous height. The chlorides fell rapidly to 0·146 per cent., showing that there was a partial recovery of the normal power of reabsorption of chlorides. The uric acid content of the urine, however, remained almost unchanged: only during the last period of the experiment, when the urinary flow slowed, did the uric acid rise to 8 mg. per cent.

Another experiment of the same nature, but with variation in detail and giving, therefore, somewhat different results, is represented in Table V and fig. 2. In this case a larger dose of uric acid was employed and a shorter period allowed for its elimination by the perfused kidney before the institution of the circulation

Table V.—25.3.26. Heart-Lung-Kidney.

Time. mins.	Art. Pr. mm. Hg.	Blood flow, Kidney, cubic centimetre per min.	Urine flow, cubic centimetre per min.	Uric Acid.			NaCl. Urine g. per cent.	NaCl. Serum g. per cent.
				Blood mg. per cent.	Urine mg. per cent.	Urine abs. am. mg.		
1.05	110	143	1·00	0	8·00		0·150	0·530
1.20	110	187	0·66		7·50		0·152	
1.21	Addition of 200 mg. Uric Acid.							
1.26	110			8·42				0·540
1.34	,,	230	0·76		85·75	8·6	0·152	
1.44	,,	,,	1·00		98·35	9·8	0·175	
1.45	,,	,,	1·00		98·35	9·8	0·159	
2.00	,,	,,	1·00		103·45	5·4	0·164	
2.01	Perfusion with M/200 Sodium-cyanide Blood.							
2.10	110	228	1·00	6·40	28·57	1·2	0·477	0·528
2.17	,,	228	0·50		18·75	0·5	0·527	
2.18	Again perfused by the Heart-Lung preparation.							
2.30	110	200	0·60	6·96	14·29	0·7	0·425	0·528
2.37	,,	,,	1·42		11·54	1·2	0·467	
2.41	,,	,,	2·00		12·50	1·3	0·370	
2.45	,,	,,	2·50		11·54	1·2	0·376	
2.55	,,	,,	2·00		11·54	2·3	0·292	
2.59	,,	,,	2·50		12·50	1·3	0·306	
3.05	,,	,,	1·67	5·92	9·38	0·9	0·294	

The preparation started at 12.38; up to 1.05 12 c.c. of urine had been secreted.

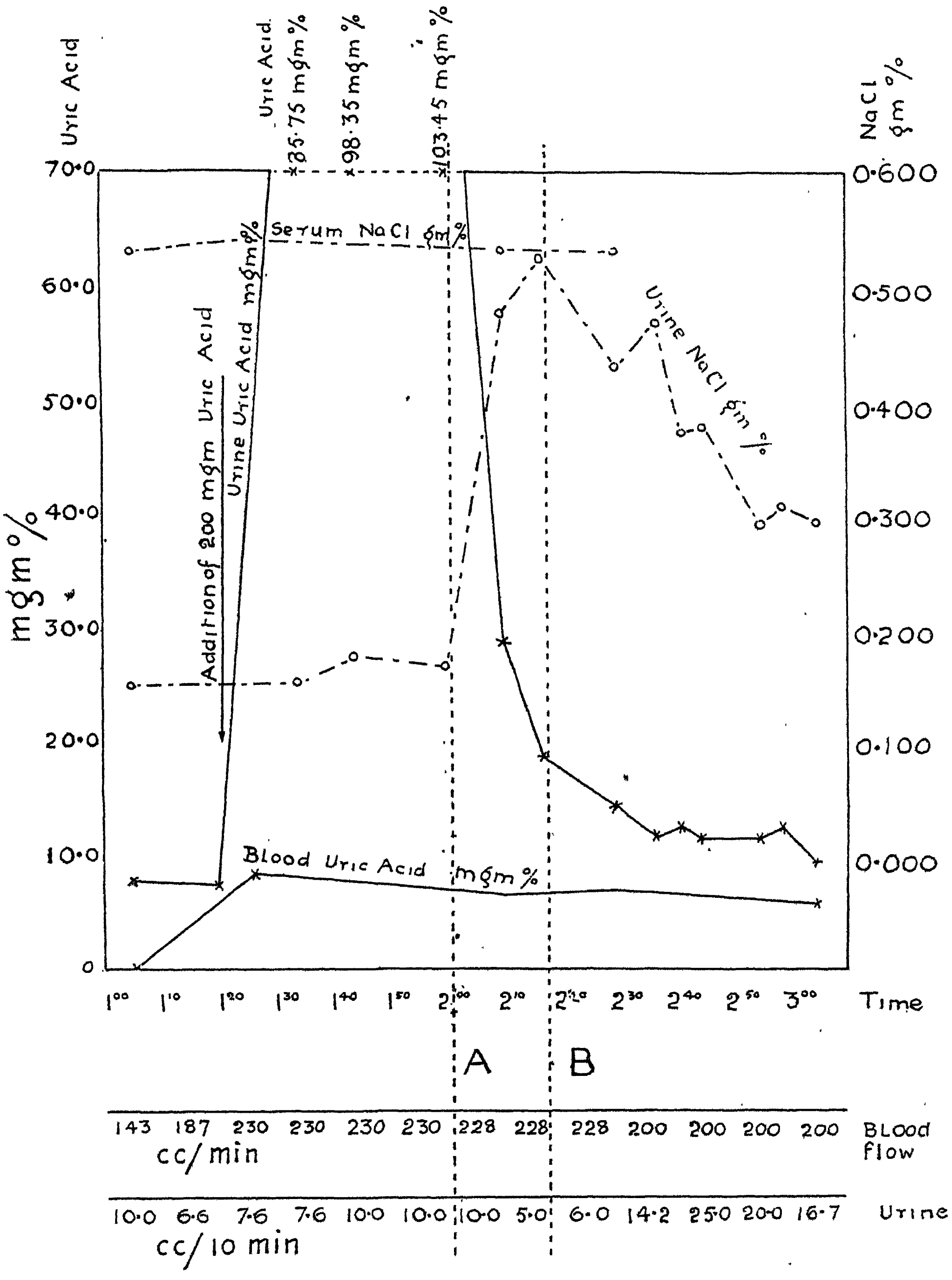

FIG. 2.—Graph of uric acid and chloride percentages in blood, blood serum and urine. Between A and B, kidney perfused with cyanide blood.

of cyanide blood. The addition of 200 mg. of uric acid raised the blood content of this substance to 8·42 mg. per cent., while the uric acid of the urine rose from 7·5 mg. to 103 mg. per cent. in about 40 minutes. The chlorides of the urine had an average value of 0·16 per cent. The blood flow during the last half-hour was constant at 230 c.c. per minute and the blood pressure at 110 mm. Hg. There was a slight increase in the urinary flow after the addition of uric acid: the flow then remained constant during the next half-hour. On circulating the cyanide blood the uric acid in the urine dropped at once to 28 and then to 18 mg. per cent., while the chlorides reached the blood serum level—namely, 0·527 per cent. During this time the level of the uric acid in the blood was 6·4 mg. per cent., so that although there was a great fall in the uric acid excretion it was still three times as concentrated in the urine as in the blood. After 17 minutes' perfusion with the cyanide blood the kidney was switched over once more to the heart-lung preparation. The blood flow and the blood pressure remained practically constant throughout the experiment. There was, however, only a slight recovery in the reabsorption of chlorides and no apparent recovery in the excretion of uric acid, the amount of which in the urine steadily diminished during the ensuing three-quarters of an hour.

Certain conclusions can be drawn from these two experiments. In the first place, we would call attention to the fact that suppression of the functions of the tubules by the circulation of cyanide blood does not alter appreciably the total amount of urine secreted. Previous to the circulation of the cyanide blood the uric acid in the process of excretion into the urine had undergone a concentration varying from three to twelve times. If this concentration had been effected merely by the absorption of water from the glomerular filtrate, as is required by Cushny's "modern theory," a suppression of the absorbing functions of the tubules should have left the total uric acid excreted per unit of time unchanged, but have increased the total quantity of urine from three to twelve times. Neither of these results took place; the total fluid excreted remained almost the same as before, while the uric acid dropped both in percentage and in total amount. In our experiments, as in those of Starling and Verney, the paralysis of the absorptive functions of the tubules under the action of cyanide is demonstrated by the attainment of equality between the chlorides of the blood serum and of the urine.

The behaviour of uric acid in these experiments indicates that in the kidney perfused with normal blood this substance was being added to the glomerular filtrate by a process of active secretion by the cells of the tubules. Its behaviour, however, does not conform exactly to any one of the substances for which a similar process of secretion has been concluded by Starling and Verney as a result of their experiments. These observers found that the urea and the sodium sulphate of the urine became immediately identical with that of the blood serum as soon as the activity of the cells of the tubules had been suppressed by cyanide, thus indicating that, under normal circumstances, no storage of this substance in the cells of the tubules took place as a preliminary to its secretion into the lumen of the tubule. This conclusion agrees with the results of Marshall (9), who, in the mammal, was unable to find any evidence of an accumulation of urea in the kidney. On the other hand, when phenol red is added to the blood in the heart-lung preparation, it is excreted by the kidney also in a concentrated form, and this excretion is associated with an accumulation of dyestuff in the kidney. If under these circumstances the activity of the tubules is suppressed by cyanide, the first amounts of urine obtained show a concentration of the dyestuffs greater than that of the blood, but this concentration diminishes with continuance of the cyanide perfusion until it becomes equal to that of the glomerular filtrate, *i.e.*, to the concentration of the filtrable part of the pigment contained in the blood serum. This was explained by Starling and Verney as due to the fact that the first portions of the glomerular filtrate formed after the suppression of the tubular functions dissolved, or leached out, from the cells of the tubules the pigment these cells had accumulated during the preceding normal period.

We have already seen that uric acid does not accumulate to any large extent in the cells of the kidney, though a certain accumulation is shown by the presence of the granules of this substance, which can be seen, after its injection, in the cells of the convoluted tubules. In the experiment given in Table IV. the kidney had excreted the uric acid during the normal period at a considerable rate, diminishing the blood content from 8·6 to 5·6 mg. per cent. The circulation of cyanide blood brought the urinary uric acid at once almost to the blood level which is attained within 15 minutes. In the experiments given in Table V, a shorter period was allowed for the elimination of the uric acid. The uric acid of the urine rose steadily from 85 to 103 mg. per cent. during the normal period. When the perfusion of cyanide blood was started, the urinary uric acid fell at once to 28 mg. per cent., and then slowly during the rest of the experiment, first to 18 and, finally, to 9·3 mg. per cent. If we take into consideration the fact that the method used gives only about 70 per cent. of the uric acid in the blood serum, this last figure does not differ appreciably from the 6 mg. per cent. found in the blood at the end of the experiment. In this case, it would seem that there was a greater accumulation of uric acid in the cells, so that the behaviour of this substance on perfusion with cyanide blood resembled more closely that described by Starling and Verney in the case of the excretion of phenol red.

We would conclude, therefore, that, as indicated by microscopic investigation, there is a certain accumulation or storage of uric acid in the cells of the convoluted tubules before its secretion, the extent to which the accumulation takes place depending on the rate at which the uric acid is turned out into the tubule and eliminated from the body. In the mechanism of its excretion uric acid thus takes a position midway between substances such as urea and sulphates, for which no accumulation can be demonstrated in the mammalian kidney, and phenol red and possibly other pigments, which accumulate to a considerable extent in the cells of the convoluted tubules in the complete absence of any glomerular filtration (as shown by the low-pressure perfusion experiments).

The fact that there was no appreciable recovery of the power of excreting uric acid, as apart from its filtration through the glomeruli, seems to indicate that this excretory function is more susceptible to the effects of cessation of the oxidative processes of the cell than is the function of absorption of chlorides. In both experiments quoted there was a considerable recovery in the power of the kidney tubules to reabsorb chlorides, but practically none in their secretory powers for uric acid. This difference in the behaviour of the two functions may point to their being localised in different cell constituents of the tubules.

The Effect of Uric Acid when Injected into the Intact Dog.—Wiechowski (10) has shown quite clearly that the end product of purine metabolism in most animals is allantoin, and that only in man and the anthropoid apes does it consist of uric acid. (The Dalmatian hound has also been described as excreting chiefly uric acid.) Wiechowski's conclusions have been confirmed by Hunter and his co-workers (11). Folin, Berglund and Derik (12) state, however, that uric acid when injected intravenously disappears very rapidly, this disappearance being due to its oxidation but not to its conversion into allantoin. They believe that the destruction of uric acid is due to the action of the blood in combination with the tissues, and that no special organ, such as the liver, plays a part in the process. They maintain that the excretion of the uric acid is of little importance, since in their experiments injection of large amounts of uric acid caused almost complete cessation of urinary flow and œdema of the kidneys. The discrepancy between Folin's results and those obtained by other observers made it necessary to repeat the experiments. The following procedure was employed :—

The dog being anæsthetised by chloralose, cannulæ were placed in both ureters by the extraperitoneal method. In our first experiments 100 mg. of uric acid per kilo were injected into the jugular vein, and the urine was collected for ten minutes; the animals were then killed and the kidneys excised for analysis. During the injection itself we noticed a large increase in the urinary flow, in one experiment from 1·1 c.c. to 4·7 c.c., in the second from 4 to 23 c.c. in ten minutes. In the first experiment we noticed that the urine passing down the cannula contained a bulky precipitate. This precipitate was identified as a compound of uric acid. The percentage of uric acid in the urine rose from 25 mg. per cent. to the enormous figure of 1,388 mg. per cent. The right kidney contained 124 mg. per cent., the left 100 mg. per cent., and the amount secreted in 10 minutes was 130 mg., being 8·1 per cent. of the total quantity injected. In the second experiment of the same type, in which the diuretic action of the uric acid was more pronounced, the uric acid concentration rose from 26 to 295 mg. per cent., and the total amount excreted in the ten minutes was 136 mg. or 9 per cent. of the total amount injected.

We have never seen the swelling of the kidneys described by Folin, and we are inclined to believe that this must have been due to the hypotonicity of the uric acid solutions he employed. As already mentioned, we took care to raise the tonicity of our solutions to that of the blood by the addition of glucose.

In our next experiments we determined the uric acid in the blood, and both the uric acid and the allantoin in the urine. The results are given in Table VI. The right kidney was extirpated ten minutes after the injection of uric acid, and contained 110 mg. per cent. uric acid. The experiment was continued with the left kidney, which at the end of the experiment contained 12 mg. per cent. of uric acid. In the urine the uric acid rose from 15 mg. per cent. to 157 mg. per cent., and then during the next two hours gradually fell to 24 mg. per cent. The blood contained 18 mg. per cent. immediately after the injection; then there was a fall, especially rapid during the first quarter of an hour; after two hours the blood uric acid was only 1·1 mg. per cent. The allantoin of the urine was 0·233 per cent. before the injection and 0·413 per cent. in the total urines collected after the injection. We performed other experiments of this kind with similar results.

There can be no doubt that uric acid is oxidised in the intact animal, though we do not believe that the rapid disappearance during the first five or ten minutes is due to oxidation. The increase of the allantoin in the urine shows that it is the chief oxidation product. The excretion of uric acid during the first ten minutes can be very extensive, and a very high concentration of this

Table VI.—20.11.25. Dog—Weight, 8 kg.

Time. mins.	Urine, Left Kidney, cubic centimetre per min.	Uric Acid.			Allantoin g. per cent.
		Blood mg. per cent.	Urine mg. per cent.	Urine abs. amount.	
Urine from the bladder					0·233
12.11	0·15				
12.21	0·16	0	15·8		
12.31	100 mg. per kg. Uric Acid injected into the jugular vein.				
12.35		18·5			
12.41	0·95	9·8	157·9	10·26	
12.46	Right Kidney removed.				
12.50	0·78		41·4	2·9	
1.10	0·25	3·9	46·2	2·6	
1.20	0·40		54·6	2·2	
1.30	0·36		35·9	1·3	
1.40	0·25	2·6	31·7	0·8	
2.00	0·22		39·2	1·8	
2.20	0·15		24·0	0·7	
2.40	0·10	1·1	24·3	0·5	0·413

Right kidney taken out 10 minutes after the injection of uric acid contained 110 mg. per cent. uric acid; the left kidney taken out 2 hours and 10 minutes after the injection contained 12·3 mg. per cent.

Uric acid excreted 23·1 mg.; 2·9 per cent. of the injected amount of uric acid.

Allantoin excreted 234 mg.; 29·2 per cent. of the injected amount of uric acid.

substance may be attained in the urine. As we have already shown for the isolated kidney, there is no evidence of a large accumulation of uric acid in the cells of the tubules, the amounts that we have found in the kidney being readily explained by the high percentage of this substance in the urine filling the urinary tubules. We cannot, therefore, accept Folin's view that uric acid is taken up in quantity by the kidney, and then given off again to be oxidised in the blood.

Uric Acid in the Heart-Lung-Kidney-Liver Preparation.—The following experiments were performed to illustrate the part taken by the liver in the oxidation of uric acid. In their admirable work on the physiology of the liver, Mann and Magath (13) have shown that after the extirpation of this organ a measurable amount of uric acid appears in the blood, and at the same time there is a large excretion of this substance, which may rise in the urine to 600 mg. per cent. In these animals uric acid injected into the circulation is entirely excreted. If excretion be prevented by eliminating the kidneys, the blood level of uric acid rises steadily and comes into equilibrium with the tissues. These authors have no doubt, therefore, that the liver is the organ wherein uric acid is oxidised. In order to test these conclusions on a simpler preparation we perfused the kidney and liver at the same time with a heart-lung preparation. To this end, after the completion of the preparation of the heart-lung, a cannula was tied into the portal vein and another into the common bile duct, the gall bladder being tied off. The liver was then cut out and placed in a hot-water funnel over the venous reservoir, the cannula in the portal vein being connected to a tube coming from the warming coil of the heart-lung apparatus. The liver was then supplied with warm, oxygenated, defibrinated blood at a low pressure. The blood flowing from the hepatic vein dropped through the funnel into the venous reservoir, with which the funnel was connected by means of a side-tube. The blood flow from the liver could be measured by diverting it for a time from the reservoir and collecting it in a graduated cylinder. The kidney of another dog was then connected to the arterial circuit in the usual manner.

Two typical experiments of this kind, A and B, are represented in Table VII and fig. 3. If we follow the uric acid figures in the blood we see that in experiment A the addition of 300 mgms. uric acid to the 1200 c.c. of blood circulating through the preparation caused a rise in the uric acid content of the blood to 9·2 mg. per cent. The second figure given—namely, 7·28—is the percentage of uric acid in the blood leaving the liver, a difference which is outside experimental error and denotes a taking up of uric acid by the liver. In the course of thirty minutes the blood uric acid fell to 1·84 mg. per cent. There is thus a remarkable difference between this experiment and the previous ones, in which the liver was not included in the circulation. The uric acid in the urine rose from 5 mg. to 17 mg. per cent. and remained constant about this level for an hour. The amount excreted in the urine cannot, however, account for the large disappearance of uric acid from the blood, since in the course of an hour only 4·1 mg. were excreted with the urine against 96 mg. removed from the blood. After about eighty minutes the uric acid level in the blood dropped to zero, while 15 mg. per cent. were still being excreted in the urine. On the other hand, the allantoin of the urine rose from 0·073 per cent. before the addition of uric acid to 0·135 per cent. after the uric acid. This second figure is the average allantoin content of all the urine collected after the addition of uric acid. The second experiment (Experiment B) given in Table VII shows similar results. 300 mg. uric acid added to the 1300 c.c. circulating blood raised the blood level to 13 mg. per cent., while the uric acid in the urine rose from 2 mg. to 30 mg. per cent. Here also the blood leaving the liver contained 3 mgms. less than the blood entering the liver. The blood uric acid dropped to 8 mgms. per cent. in half an hour and fell continuously until it reached zero in 70 minutes. Of the 160 mg.

Table VIIA.—21.1.26. Heart-Lung-Kidney-Liver Preparation (1200 c.c. blood in circulation).

Time.	Art. Pr. mm. Hg.	Blood flow, Kidney, cubic centimetre per min.	Blood flow, Liver, cubic centimetre per min.	Uric Acid.			NaCl. Urine g. per cent.	Urine, cubic centimetre per min.	Allantoin g. per cent.
				Blood mg. per cent.	Urine mg. per cent.	Urine abs. amount.			
1.16	100	158	400	0	5·66		0·024	0·35	
1.26	100	158	400		5·46		0·024	0·40	
1.39	100	158	400		5·56		0·024	0·31	
1.51	100	158	400		5·36		0·025	0·34	0·073
1.52	300	mg. of uric acid added.							
1.56	100	201	400	9·24*a* 7·28*b*	17·65	0·7	0·070	0·8	
2.08	100	201	400		18·75	1·5	0·053	0·67	
2.22	100	187	336	1·84	20·00	1·6	0·050	0·57	
3.12	100	177	336	0	15·00	1·3	0·043	0·16	0·135

Table VIIB.—28.1.26. Heart-Lung-Kidney-Liver Preparation (1300 c.c. blood in circulation).

Time.	Art. Pr. mm. Hg.	Blood flow, Kidney, cubic centimetre per min.	Blood flow, Liver, cubic centimetre per min.	Blood mg. per cent.	Urine mg. per cent.	Urine abs. amount.	NaCl. Urine g. per cent.	Urine, cubic centimetre per min.	Allantoin g. per cent.
2.06	100	157	300		3·00		0·029	0·35	
2.18	100			0	2·43		0·027	0·32	0·158
2.19	300	mg. uric acid added.							
2.23	100			13·7*a* 10·9*b*					
2.39	100	157	300		30·00	1·7	0·015	0·29	
3.00	100	157	300	8·66	25·00	2·0	0·015	0·27	
3.15	100	157	300		28·60	1·1	0·015	0·27	
3.23	100			1·44					
3.30	100	150	260		27·27	1·1	0·029	0·27	
3.35	100	150	260	0	30·00	0·3	0·040	0·20	0·252

which had disappeared from the blood, only 6·2 mg. had been excreted with the urine. The urinary allantoin rose from 0·156 per cent. to an average of 0·252 per cent. in the total urines secreted after the addition of uric acid. In Table VIII we give the results of an experiment in which only the liver was connected with the heart-lung preparation. Here, after the addition of uric acid, the uric acid of the blood diminished from 17·8 mg. per cent. to zero in about 2 hours.

Thus in the heart-lung-liver preparation most of the uric acid is oxidised to allantoin and only a comparatively small fraction is excreted. There are thus three factors involved in the disappearance of the uric acid which Folin observed in his experiments on the whole animal and which he ascribes to oxidation in the blood. The first rapid fall is due to a distribution of the uric acid between the blood and the tissues throughout the body. After this the

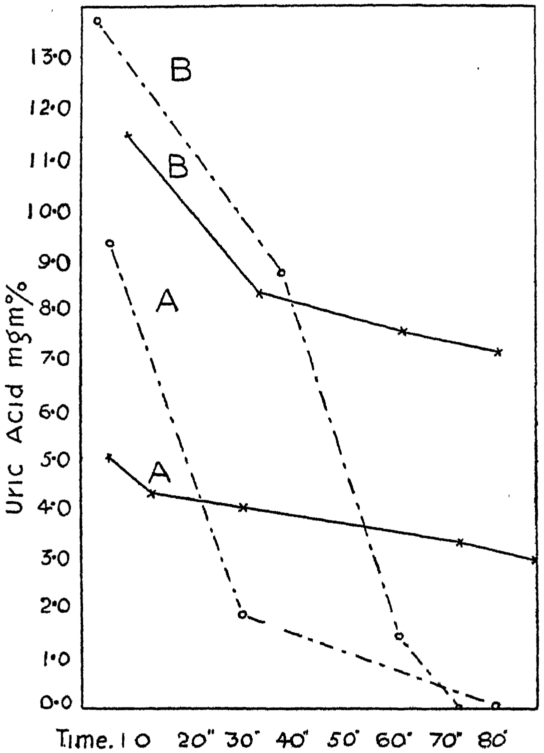

FIG. 3.—Effect of introducing the liver into the circulation on the rate of fall of the uric acid percentage in the blood. Two pairs of experiments, A and B. The continuous lines represent the uric acid percentages in the blood of two heart-lung-kidney preparations, the interrupted lines the corresponding values in two heart-lung-kidney-liver preparations.

Table VIII.—17.2.6. Heart-Lung-Liver Preparation.

Time.	Art Pr. mm. Hg.	Blood flow, Liver, cubic centimetre per min.	Uric acid, Blood mg. per cent.
1.05	100	120	0
1.15	300 mg. Uric acid added.		
1.20	100	210	17·8
1.49	100	210	11·4
2.50	100	210	3·8
3.45	100	210	0

uric acid disappears from the blood in two ways—namely, by excretion through the kidney and by oxidation in the liver. The rate of fall can be markedly diminished if the liver, therefore, be excluded from the circulation. In one experiment an Eck's fistula was made and the hepatic artery ligatured, thus shutting out the liver from the blood circuit, at any rate sufficiently for our purpose. 100 mgs. per kilo of uric acid were then injected: the first blood sample, taken after 2 minutes, contained 50 mgms. per cent., the second, after 5 minutes, 30 mgms. per cent., the third, after 20 minutes, 22 mgs. per cent., and the fourth after 45 minutes, 21 mgms. This slow fall in the uric acid content of the blood shows conclusively that in the normal animal the rapid disappearance of uric acid from the blood which occurs after the preliminary equilisation of concentrations between blood and tissues is due to the largest extent to its oxidation in the liver.

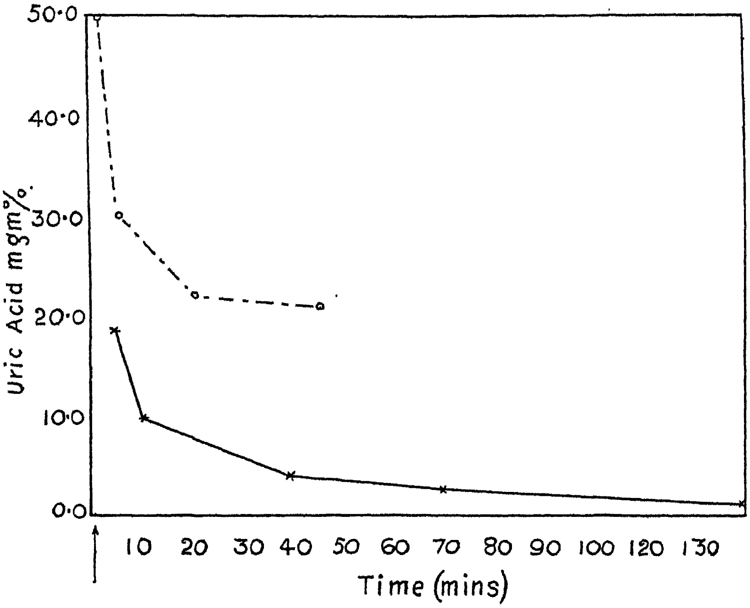

Fig. 4.—The effect of excluding the liver from the circulation on the rate of disappearance of uric acid from the blood. The continuous line represents the uric acid of the blood in milligrams per cent. in an intact animal, after its injection intravenously; the interrupted line the corresponding values in an animal in which the liver has been cut out of the circulation by an Eck's fistula.

Discussion.

If uric acid is added to the blood of a heart-lung-kidney preparation, it is excreted by the kidney. A certain relationship exists between the concentration of the uric acid in the blood and in the urine, depending upon the concentration in the blood and the rate of urinary flow. The urinary percentage is at least two or three times greater than that of the blood, and may attain a value ten to twelve times that in the blood.

When uric acid is injected into the whole animal, the concentration of the uric acid in the urine may even exceed its solubility, so that this substance is precipitated in the urine as it passes down the ureter cannula. Neither in the isolated kidney nor in the whole animal did we notice any injurious effects on the kidney; on the contrary, uric acid acted in most cases as a diuretic. We are inclined to ascribe the injurious effects observed by Folin to the hypotonic character of his uric acid solutions. In our experiments this difficulty was avoided by the addition of 4 per cent. glucose to these solutions. In the heart-lung-kidney preparation all the uric acid which disappears from the blood can be recovered in the urine, the blood level of the uric acid dropping gradually as the uric acid is excreted, the rate of fall depending on the rate of urinary flow. In our low-pressure experiments, when the secretion of urine was prevented by reducing the blood pressure to 35 mm. Hg., the percentage of uric acid in the blood remained constant. No evidence was ever obtained of the oxidation of uric acid occurring in the blood. The suppression of the glomerular and tubular functions of the kidney by the cyanide method shows that uric acid is actually secreted into the glomerular filtrate by the cells of the tubules.

Starling and Verney showed that two types of secretory activity could be distinguished in the kidney, represented respectively by the behaviour of urea and phenol red. Urea is taken out of the blood and immediately transferred in a greater concentration to the lumen of the tubules. There is thus no storage of urea in the kidney, and after the application of cyanide, the urea of the urine falls suddenly to the blood level. On the other hand, phenol red is removed from the blood by the tubular cells even when no glomerular transudation is occurring, as in the low-pressure experiments. It is stored up in these cells, and when a kidney in which this storage has occurred is perfused with cyanide blood, the concentration of the phenol red in the urine is at first greater than its concentration in the blood, owing to the leaching out of the dye from the paralysed cells of the tubules. When this store is exhausted, the concentration of the dyestuff in the urine falls to that of the glomerular filtrate. Uric acid seems to occupy a position midway between these two classes of substances. The low-pressure perfusion experiments indicate no considerable storage of this substance in the kidney. On the other hand, the perfusion of the kidney with cyanide soon after the addition of a large amount of uric acid to the circulating blood, though causing a drop in the concentration of the uric acid excreted, did not bring this figure down to the level of that in the blood, but the uric acid concentration fell gradually somewhat in the same way as had been observed for the phenol red. There thus appears to be a certain, but limited, storage of uric acid in the cells of the tubules prior to its excretion into the lumen, and this conclusion is confirmed by the observation which has been frequently made of granules of uric acid in these cells as the result of uric acid injection into the blood.

We are also able to confirm the statements of Starling and Verney with regard to the secretion of chlorides. These are filtered off by the glomeruli, and are then reabsorbed to a large extent during the passage of the filtrate down the tubule. On paralysing the tubular cells by cyanide this reabsorption is abolished, and the chloride level of the urine rises to that of the blood serum.

The secretory and reabsorptive functions of the tubular cells show different sensitivities to the action of cyanide. When the circulation of the cyanide blood is replaced by that of normal blood, there is a certain amount of recovery of the power to reabsorb chlorides, but practically no recovery of the secretory function for uric acid. In the heart-lung-kidney preparation there is no evidence of any oxidation of the uric acid. If, on the other hand, the liver is included with the kidney in the circulation, there is a marked and rapid oxidation of the uric acid in the circulation. The product of this oxidation is allantoin. In the heart-lung-kidney preparation the allantoin of the urine diminishes continuously throughout the experiment, whereas if the liver be included, the addition of uric acid to the circulating blood causes a marked rise in the allantoin which appears in the urine.

Our experiments thus confirm the conclusions of Mann and Magath that the liver is the only organ concerned in the oxidation of uric acid, and our experiments confirm, moreover, the accepted view that allantoin is the product of the oxidation of uric acid.

We found no evidence for Folin's statement that the kidney removes the uric acid from the blood and then returns it to this fluid for oxidation to take place in the blood itself. The rapid initial disappearance of uric acid is not due to its rapid destruction, but to the establishment of an equilibrium between its concentration in the blood and in the tissues. The later, more gradual, disappearance of uric acid is due to its oxidation in the liver, and to a less degree to its excretion by the urine. In the whole animal, uric acid injected into the blood disappears by oxidation in about one hour; in the isolated heart-lung-liver-kidney preparation, this disappearance is complete in eighty minutes.

Conclusions.

(1) Injected uric acid is excreted by the isolated perfused kidney.

(2) The concentration of the uric acid in the urine is dependent upon the concentration of uric acid in the blood and also upon the rate of urine flow.

(3) Uric acid both in the isolated kidney and in the intact animal has a more or less pronounced diuretic effect.

(4) Uric acid is actually secreted by the tubular cells of the kidney.

(5) In the intact animal the uric acid is mainly oxidised rather than excreted, the former occurring to a far greater extent than the latter. This is also true for the heart-lung-liver-kidney preparation.

(6) The oxidation of uric acid takes place in the liver.

(7) The oxidation product of uric acid is allantoin.

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The Isolation of some hitherto Undescribed Products of Hydrolysis of Proteins.—Part III.

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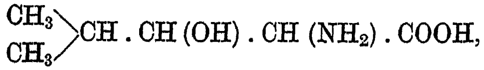
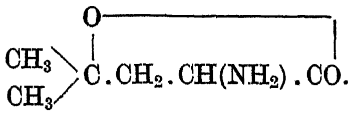
The recent discoveries of hydroxylysine, hydroxyvaline, and hydroxyamino-butyric acid, described in the earlier papers of this series, together with the discovery a few years earlier of hydroxyglutamic acid by Dakin, lead to the conception that there is to be found among the hydrolysis products of the proteins a hydroxyl derivative corresponding to every (or nearly every) non-hydroxyamino-acid that has been isolated. Yet, so far, the hydroxyl derivative of leucine, perhaps the most widely distributed hydrolysis product of the proteins, has not been discovered.

In searching for such a substance, certain possibilities of chemical change must be taken into account. It is known that in the presence of hot solutions of strong acids, such as the 25 per cent. sulphuric acid used for the hydrolysis of proteins, hydroxy derivatives of long-chain substances tend to undergo isomeric change, with the wandering of the hydroxyl group from one position to another. Supposing, now, that hydroxyleucine exists among the hydrolysis products of proteins, and that the hydroxyl group is in the β position,



$$\begin{matrix}CH_3\\CH_3\end{matrix}\!\!>CH\,.\,CH\,(OH)\,.\,CH\,(NH_2)\,.\,COOH,$$

then, in the presence of hot acid solutions, it is conceivable that the hydroxyl group wanders to the γ position, and a lactone would thereby be obtained of the formula



$$\begin{array}{l}\qquad\;\; O\text{—————————}\,|\\ \qquad\;\;|\qquad\qquad\qquad\qquad\quad\;|\\ \begin{matrix}CH_3\\CH_3\end{matrix}\!\!>C\,.\,CH_2\,.\,CH(NH_2)\,.\,CO.\end{array}$$

A similar lactone might be obtainable from hydroxyvaline.

Now such substances would be sufficiently basic to be precipitable by phosphotungstic acid, and would also, probably, be soluble in alcohol and other organic solvents (*cf.* the properties of the amino-butyrolactone, prepared synthetically by E. Fischer*).

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* 'Berichte,' vol. 40, p. 106 (1907).

In view of the above considerations it was thought advisable to re-examine the basic fractions obtained by the hydrolysis of proteins. So far these have been examined in the case of two proteins only, viz., the protein of castor bean and the glutelin-like protein of oats. As a result, no amino-lactones have been isolated, but in both instances a base, hitherto unknown, has been discovered, having the formula $C_8H_{15}O_3N_3$. Its constitution has not been determined, and the name suggested for it is "protoctine," to indicate a base with eight carbon atoms, derived from proteins. The isolation of this substance from two proteins of such different origin suggests that it may be very widely distributed; further work will be undertaken to determine this.

Preparation of the Base.

As stated above, the base has been isolated from the hydrolysis products of the glutelin-like protein of oats, and of the alkali-soluble protein of castor bean. The preparation of the oat protein was described in detail in an earlier paper*; the castor-bean protein was prepared by extracting the oil-free castor beans with 0·2 per cent. sodium hydroxide, clarifying the extract by passing it through a Sharples centrifuge, and precipitating the crude protein by means of acetic acid. The protein was further purified by redissolving in 0·2 per cent. alkali, filtering through paper pulp, and reprecipitating with acid; it was washed with graded strengths of alcohol, and finally with ether, and dried.

The method of isolating the base was precisely the same in the case of each protein. The protein was hydrolysed by sulphuric acid, and after the removal of sulphuric acid the dicarboxylates were precipitated with barium hydroxide and alcohol, and the filtrates were then converted into the barium carbamates by the methods described in the earlier papers. These were then extracted with ice-cold water, which does not extract the barium carbamate of hydroxylysine. The solution of the soluble carbamates was decomposed by boiling, liberating thus the free amino-acids. To the amino-acid solution, phosphotungstic acid was added to precipitate the basic fraction. This was then decomposed in the ordinary way with barium hydroxide.

The solution of the free bases was evaporated to small bulk, care being taken to avoid the formation of carbonate, and the somewhat syrupy liquid was taken up on plates of porous porcelain. The plates were dried as completely as possible *in vacuo* over solid sodium hydroxide, after which they were broken up and extracted by boiling under reflux with absolute alcohol for one hour. The extraction was repeated twice, care being taken throughout to exclude carbon dioxide.

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* 'Roy. Soc. Proc.,' B, vol. 99, p. 476 (1926).

The first extract was fairly strongly coloured, the second almost colourless; a small amount of solid separated on cooling the extract, and was removed. The alcohol was distilled off *in vacuo*, and the residual syrup taken up in water; to remove any arginine or histidine, which might be expected to be present, the usual method of precipitation with silver sulphate and barium hydroxide was adopted. In the case of the product from oats, 12 per cent. of the nitrogen present was precipitated at this stage; in the case of the castor bean, 13 per cent. was precipitated.

The filtrate, after removal of silver, etc., was evaporated to a syrup, which was taken up with absolute alcohol; the alcoholic solution was repeatedly evaporated and re-dissolved till finally a product was obtained that was entirely soluble in absolute alcohol, and was not precipitated on addition of more alcohol. From the castor-bean product, residues insoluble in alcohol at this stage were obtained, amounting to 3·0 gm. and containing 17·37 per cent. of nitrogen (lysine); a corresponding amount was obtained in the case of the oats.

Finally, the clear alcoholic solutions were evaporated to dryness and granulated with dry ether. From 1400 gm. of the castor-bean protein 20 gm. of the product were obtained, and from 700 gm. of the oat protein 4·5 gm. These yields correspond respectively with 2 per cent. and 0·5 per cent. of the total nitrogen in the protein. The base was in each case further purified by recrystallisation of the picrate (see below), and dried *in vacuo* at 100° over phosphorus pentoxide and solid caustic potash.

Properties and Analysis of the Base.

The base, as obtained by the above method, was a cream-coloured powder, very soluble in water, readily soluble in absolute alcohol, and insoluble in ether. It was extremely deliquescent; its aqueous solution was alkaline in reaction and absorbed carbon dioxide from the air. The solution of the base decolorised permanganate in acid solution, and gave certain colour reactions which are described below. It was precipitated from solution by mercuric chloride and barium hydroxide, but not by silver nitrate and alkali. The free base had no definite melting-point, but darkened, and finally charred above 220° C. The identity of the samples of base from the two different sources was established by elementary analyses and by the properties of their benzoyl derivatives.

Analysis of the free base gave the following figures :—

(i) 0·004022 gm. gave 0·007004 gm. CO_2 and 0.002888 gm. H_2O.
(ii) 0·003114 gm. gave 0·005435 gm. CO_2 and 0·002116 gm. H_2O.
(iii) 0·002846 gm. gave 0·004974 gm. CO_2 and 0·001917 gm. H_2O.

Hence C = 47·49 per cent., 47·59 per cent., 47·67 per cent.
H = 7·95 per cent., 7·55 per cent., 7·78 per cent.
N = 20·61 per cent., 20·65 per cent. (Kjeldahl).

(Combustions Nos. (i) and (ii) on the castor-bean product, No. (iii) on that from oats.)

These figures agree with an empirical formula $C_8H_{15}O_3N_3$, which requires C = 47·77 per cent., H = 7·46 per cent., N = 20·89 per cent.

(The figures for hydrogen tend to become somewhat high, owing to the very deliquescent nature of the base.)

The molecular weight was determined by estimating the depression of freezing-point in aqueous solution. 0·785 gm. of base dissolved in 50 c.c. water gave a depression of 0·152° C. Hence, molecular weight = 192·1. The formula given above requires a molecular weight of 201; the molecule would, therefore, appear to be represented by the simple formula and not by a multiple of it.

Titration by alkali in the presence of formaldehyde gave anomalous results, two determinations giving equivalents of 425 and 433 respectively; the reason for this anomaly is discussed below. Titration of the alcoholic solution with alcoholic caustic soda (under which conditions amino-groups do not give rise to free hydroxyl ions) gave a normal result, using phenolphthalein as indicator :—

0·126 gm. required 6·03 c.c. 0·1 N caustic soda, giving an equivalent weight = 209.

Further evidence concerning molecular weight was obtained by analysis of the picrate and of the copper salt (see below).

The reaction between nitrous acid and the base was investigated to determine the proportion of the nitrogen in the amino form; 35·1 per cent. of the total nitrogen was found as amino-nitrogen; this was not increased by prolonged hydrolysis. It was, therefore, concluded that only one of the three nitrogen atoms was in the amino form; this supposition is confirmed by the preparation of the phenyl isocyanate derivative. The fact that the substance is fairly strongly basic indicates that the other two nitrogens are present in some group that exercises basic functions (*cf.* histidine, in which two of the three nitrogens are present in the iminazole ring).

Determination of Dissociation Constants.

An electrometric titration of the base was carried out, a known excess of acid being added, and back-titrated over a wide range of p_H. It was found impossible to deduce basic dissociation constants from the figures obtained, no constant agreeing with all points on the curve. Presumably, there are two weak basic groups, with basic dissociation constants not far removed from one another, and these produce a combination curve.

The portion of the curve concerned with the dissociation of the carboxyl group was normal, and an acid dissociation constant of $K_a = 1 \cdot 8 \times 10^{-13}$ was deduced.

(The thanks of the authors are due to Dr. H. T. S. Britton, who kindly carried out this experiment and compiled the table given below.)

To 137·1 c.c. of 0·0981 M solution of the base (p_H 9·38):—

Cubic centimetres of 0·0918N NaOH added.	E.M.F.	p_H	Conc. OH′ ions.	Conc. added NaOH.	Conc. NaR.	Free acid.	$K \times 10^{-13}$
6·9	0·934*v*	11·23	$1 \cdot 23 \times 10^{-3}$	$4 \cdot 37 \times 10^{-3}$	$3 \cdot 14 \times 10^{-3}$	$90 \cdot 3 \times 10^{-3}$	2·04
8·9	0·951*v*	11·52	$2 \cdot 40 \times 10^{-3}$	$6 \cdot 76 \times 10^{-3}$	$4 \cdot 36 \times 10^{-3}$	$86 \cdot 6 \times 10^{-3}$	1·91
50·0	0·956*v*	11·61	$2 \cdot 95 \times 10^{-3}$	$7 \cdot 89 \times 10^{-3}$	$4 \cdot 94 \times 10^{-3}$	$84 \cdot 6 \times 10^{-3}$	1·43

Hence mean $K_{acid} = 1 \cdot 8 \times 10^{-13}$.

(*Cf.* K_a of arginine, given by Harris* $= 1 \cdot 4 \times 10^{-13}$).

Harris* showed that the Sørensen formalin titration of amino acids depended on the fact that the introduction of the methylene group into the molecule enhanced the dissociation constant about a thousand-fold and, consequently, rendered the acid titratable to phenolphthalein. He experimented with acids whose original constants were not less than 10^{-11}, but in the case of the acid under consideration, it follows that the dissociation constant of the methylene derivative formed by the addition of formaldehyde will be of the order of 10^{-10}, and, therefore, the p_H ruling at the end of the titration will be below that at which phenolphthalein reddens. These considerations furnish an explanation of the anomalous results given by the formalin titration and referred to above.

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* 'Roy. Soc. Proc.,' B, vol. 97, p. 364 (1925).

Derivatives of the Base.

The *hydrochloride* and *nitrate* could not be obtained in crystalline form.

The *aurichloride* crystallises in the form of golden-brown needles, and the *platinichloride* in yellow needles.

The *picrate* is readily prepared by addition of alcoholic picric acid to an alcoholic solution of the base; it may be recrystallised from hot 50 per cent. alcohol, separating first as an oil, which forms a crystalline mass on standing some hours. It decomposes at 205°–210° C. without melting. Picric acid was estimated by direct precipitation with the "nitron" reagent:—

(i) 0·03046 gm. of picrate gave 0·03828 gm. nitron picrate.
(ii) 0·02952 gm. of picrate gave 0·03683 gm. nitron picrate.

Hence equivalent of the base = 201·5; 204·6.

Copper Salt.—By boiling an aqueous solution of the base with fresh copper hydroxide a deep blue solution was obtained; on evaporation this did not crystallise, but set to a glassy mass. It was insoluble in methyl and ethyl alcohols. Copper was estimated by combustion of the salt in oxygen and weighing the residue of cupric oxide (Pregl):—

(i) 0·002285 gm. of salt gave 0·000394 gm. of cupric oxide.

Hence percentage of Cu = 13·78 per cent., and equivalent of base = 198·9.

Phenyl Isocyanate derivative.—This was prepared by shaking 0·5 gm. of the base, dissolved in 15 c.c. N.NaOH, with 1 c.c. phenyl isocyanate, and precipitating with acid. The product was recrystallised from dilute alcohol, and had a melting-point of 130° C. (with darkening).

The derivative was analysed by micro-combustion:—

(i) 0·002741 gm. gave 0·005701 gm. CO_2 and 0·001604 gm. H_2O.
(ii) 0·003386 gm. gave 0·007002 gm. CO_2 and 0·001998 gm. H_2O.

Hence C = 56·70 per cent., 56·39 per cent.
H = 6·49 per cent., 6·56 per cent.
N = 17·40 per cent.

Required for $C_{15}H_{20}O_4N_4$:—

C = 56·26 per cent.; H = 6·26 per cent.; N = 17·51 per cent.

Phenyl Hydantoin.—The phenyl isocyanate derivative was evaporated to dryness on the steam-bath with concentrated hydrochloric acid, thus being converted to the phenyl hydantoin. This was recrystallised from alcohol and melted at 148° C. Analysis: 0·002398 gm. gave 0·005220 gm. CO_2 and 0·001338 gm. H_2O.

Hence C = 59·37 per cent.; H = 6·20 per cent.

Required for $C_{15}H_{18}O_3N_4$:—C = 59·6 per cent.; H = 5·96 per cent.

Hydantoin derivative.—Attempts to prepare the hydantoin by the action of potassium cyanate were unsuccessful. The product was obtained in very small yield as a sticky mass which did not crystallise.

Benzoyl derivative.—The base was benzoylated by the ordinary Schotten-Baumann reaction in alkaline solution, and the benzoyl compound, together with benzoic acid, were precipitated by acidifying. Benzoic acid was removed by thorough washing with hot water, and the benzoyl compound was recrystallised from dry benzene, when it was obtained in the form of fine white needles—melting-point 109° C.

Combustion of the derivative gave the following results:—

(i) 0·002824 gm. gave 0·006717 gm. CO_2 and 0·001472 gm. H_2O.
(ii) 0·002708 gm. gave 0·006422 gm. CO_2 and 0·001438 gm. H_2O.
(iii) 0·003828 gm. gave 0·342 c.c. nitrogen at 16° C. and 745 mm.

Hence C = 64·84 per cent.; 64·63 per cent.
H = 5·79 per cent.; 5·89 per cent.
N = 10·21 per cent.

These figures agree with a dibenzoyl derivative, which requires:—

C = 64·54 per cent.; H = 5·63 per cent.; N = 10·27 per cent.

Thus it would appear that the third atom of oxygen in the molecule of the base is in the form of a hydroxyl group, benzoylation occurring at this group and at the amino group.

Various Colour Reactions.

1. An alkaline solution of the base gives with diazobenzenesulphonic acid an orange-red colour, changing to orange-yellow on addition of acid; on reduction with zinc dust and addition of ammonia the colour becomes light brown, and is changed by hydrogen peroxide to a very faint pinkish-brown. (Histidine under the same conditions gives a cherry-red colour, changing to orange on acidifying, golden-brown after reduction and addition of ammonia, changing to a characteristic lemon-yellow with hydrogen peroxide.*)

2. Histidine also gives a characteristic reaction with bromine water; in

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* Totani, 'Biochem. J.,' vol. 9, p. 385 (1915).

acid it gives a deep wine-red colour on warming, together with an amorphous black precipitate. In the case of the present base bromine water gives a flocculent yellow precipitate, which is destroyed on warming, the solution becoming colourless; in acid solution the yellow colour is similarly destroyed on warming. The precipitate produced by bromine water rapidly settles down to a sticky mass; some attempt was made to isolate this compound in a crystalline form, but without success.

Estimation of Methylimido Groups.

The empirical formula of the base suggested that it might be a hydroxyl derivative of a reduced alkylated histidine, and attempts were therefore made to ascertain whether any of the nitrogen atoms were alkylated. The base was treated with hydriodic acid at high temperature, according to the well-known method of Herzig and Meyer. No trace of alkyl groups was detected.

Summary.

The method of isolation of a base, hitherto undiscovered, from the hydrolysis products of proteins, is described; the base differs from the other known basic hydrolysis products in that it is readily soluble in absolute alcohol. It has been obtained at present from the proteins of oats and castor beans. It has the empirical formula $C_8H_{15}O_3N_3$, and beyond the fact that the molecule contains one amino group, one carboxyl group and one hydroxyl group, its constitution is unknown. The other two nitrogen atoms appear to be in the form of some group exercising a basic function; from analogy with histidine this may be a ring, similar to the iminazole ring. The base has been designated "protoctine," to indicate an eight-carbon base from proteins.

The base has an acid dissociation constant of $1 \cdot 8 \times 10^{-13}$; no basic dissociation constants could be deduced from the curve given by an electrometric titration.

The base and most of its salts are extremely soluble in water; the dibenzoyl derivative has a melting-point of 109°; the phenyl isocyanate melts at 130°, and the phenyl hydantoin at 148° C. Various colour reactions are described, by means of which the base can be distinguished from histidine.

From the fact that it is obtained from proteins of such different origin, it is not unreasonable to assume that it is widely distributed, especially as it has been found amongst the hydrolysis products of the only two proteins which have been examined in the search for alcohol-soluble bases.

Reports from the Royal Society's Kala Azar Commission in China.

No. 1.—*Serological Studies on Chinese Kala Azar.*

By Edward Hindle, M.A. (Camb.), P. C. Hou and Major W. S. Patton, I.M.S. (Retd.), Kala Azar Commission of the Royal Society (N. China).

(Received May 25, 1926.)

With the object of improving the present methods of diagnosis and obviating the necessity for spleen or liver puncture, various experiments have been made to try and determine the serological changes that take place in the blood of Kala Azar patients. Up to the present, only two methods have been found to give satisfactory results, viz., the "aldehyde reaction," introduced by Napier (1921), and the "globulin precipitation test," discovered by Brahmachari (1917), and modified by Ray (1921).

Both these methods are extensively used in China as an aid in the diagnosis of Kala Azar, and in some hospitals liver or spleen puncture is no longer practised. Struthers and Ch'i (1924) published the results of the examination of 141 Kala Azar patients, employing these methods of diagnosis, and found that 92·9 per cent. gave a positive globulin precipitation test, and 98 per cent. a positive aldehyde test. It is evident, therefore, that both these tests, and especially the latter, are a valuable aid to diagnosis. A few experiments were made to determine whether the blood of infected hamsters undergoes the same changes as take place in human cases. The results showed that infection in these animals was accompanied by similar serological changes, and consequently their blood was precipitated in the same way as that of Kala Azar patients.

It is evident, therefore, that infection with Kala Azar produces marked changes in the blood, and it is rather surprising that, up to the present, most of the usual serological studies have failed to show the existence of any specific agglutinins, lysins, precipitins, or other antibodies (*vide* Cornwall and La Frenais, 1916). In spite of these negative results, however, we have thought it desirable to make some experiments with the blood of patients, to see whether Chinese Kala Azar gives the same results as the disease in other countries.

Effect of Blood Serum on Flagellates.

The destruction of the flagellates of *Leishmania donovani* by human blood was first observed by Patton (1912), who, in the course of transmission experiments with *Cimex,* discovered that when a bug containing these flagellates was fed, the influx of fresh blood caused their disappearance. Experiments *in vitro*, made in conjunction with La Frenais, confirmed this observation that fresh human blood is inimical to the flagellates of *Leishmania donovani*. Archibald (1914), working with the Sudan strain of *Leishmania*, also found that human blood rapidly destroyed flagellates in culture. Cornwall and La Frenais (1916) investigated this problem in detail, and found that the fresh sera of man, goat, sheep and guinea-pig destroyed flagellates. In their earlier experiments the "hanging-drop" method was employed, and equal parts of culture and serum; but subsequently one part of culture was mixed with five parts of serum in a pipette, and the mixture left at room temperature for 24 hours. The inimical action of the sera was destroyed by heating to 55° C. for half an hour.

Olsen (1918) examined the sera of two cases of Kala Azar and found that both infected and normal human sera destroyed the flagellates, but no effect was obtained with the sera of mouse, rabbit, cat, dog or sheep, although a slight effect was observed when guinea-pig serum was employed. Heating for half an hour at 55° C. destroyed the inimical property of the human sera, and the subsequent addition of complement did not restore it. Curiously enough, the sera of moribund tubercular patients, and also the cerebro-spinal fluid, were found to have no effect. Knowles (1920) found that the sera of both Kala Azar patients and normal individuals immobilized and finally destroyed flagellates. Guigni, as a result of these observations, expressed the view that in making cultures of *Leishmania* from the peripheral blood, the human serum inhibited the growth of flagellates; but Jemma (1922) investigated this question and found that, as a rule, human blood does not hinder growth, although the blood of certain individuals has a retarding effect.

Pedroso (1921) working in South America, with cultures of the local strain of Cutaneous Leishmaniasis, found that the flagellates would live a few hours in human, sheep, rabbit, dog and goat serum.

It will be noticed that in the case of the Indian strain of *Leishmania*, all observations agree as to the inimical effect of normal human serum on the flagellates, and, therefore, it seemed of interest to find out whether the Chinese strain of *Leishmania* was equally susceptible.

In our experiments the hanging-drop method was generally employed, and the serum to be tested was mixed with equal parts of an active culture of *Leishmania* on Wenyon's N.N.N. medium, and kept at about 20° C.

The results obtained were extremely variable, for in some cases both normal serum and that of Kala Azar patients immobilized and destroyed the flagellates, whilst in others, employing the identical culture, the flagellates were still alive after 24 hours. Out of 40 normal human sera, 24 completely immobilized flagellates, whilst 16 failed to produce this effect, although the same proportions of culture and serum were employed; also, out of 39 Kala Azar patients, the sera of only 25 completely immobilized the flagellates. Similar inconstant results were obtained with the blood of normal and infected hamsters, and with the blood of the following animals:—dog, sheep, rabbit and guinea-pig. In every case, however, there was evidence of the immobilization and destruction of some flagellates, although there was considerable variation in the individual activity of the serum. However, by increasing the relative proportion of serum, the majority of flagellates could be immobilized. In every case, without exception, serum that had been inactivated by heating for 30 minutes at 55° C. had no effect on the flagellates, suggesting that the activity of the serum depends upon complement.

The cerebro-spinal fluid of seven patients, including one case of Kala Azar, had no effect on the flagellates, probably owing to the fact that normally this fluid does not contain any complement. It may be mentioned that the serum of the Kala Azar patient used in this experiment was very active, killing flagellates in dilutions of one in eight. In one experiment the sera of two dogs were found to have very little effect on flagellates; nevertheless, after being chloroformed, their blood was found to have become strongly active, destroying the parasites in a few minutes. Similar results were also obtained with hamsters, so it seems that the small amount of chloroform absorbed by the blood is sufficient to affect the flagellates.

The above-mentioned results, combined with those of previous workers, would seem to indicate that the flagellate stage cannot be the phase which invades the human body, for presumably any such parasites would be destroyed by the action of the serum. Further experiments, however, have convinced us that this "destruction" of the flagellates is more apparent than real. Certainly the majority of flagellates are immobilized when mixed with serum and many of them dissolved; consequently, in hanging-drop preparations, one is liable to assume that they have completely disappeared. By staining such preparations, however, we have frequently been able to find parasites, with no obvious signs of degeneration, in hanging drops that seemed to be negative; moreover, the results of culture experiments also indicate that the parasites are not completely destroyed by serum. Conclusive evidence, however, that the flagellate stage is capable of producing infection is afforded by the results of animal experiments, for hamsters were easily infected by the intraperitoneal injection of flagellates, and also in some cases by subcutaneous and percutaneous inoculations of the same phase of the parasite (*vide* Hindle and Patton, 1926).

It would seem, therefore, that the flagellates in cultures must possess biological differences, and that although the great majority of them are destroyed by serum, a few are capable of withstanding its effects, and these forms are responsible for the infection produced when flagellates are introduced into the body of a susceptible host. We have been unable, however, to detect any morphological characters associated with resistance to the effect of serum.

With the object of trying to follow the course of infection when flagellates are introduced into the body, open capillary tubes containing a rich culture of *Leishmania* were placed both under the skin and also in the peritoneal cavity of hamsters, and removed after varying intervals. In every case, as might be expected, large numbers of phagocytes invaded the tubes, and the flagellates completely disappeared, but we were unable to find phagocytes containing any stage of the parasite suggestive of development.

Amount of Complement in the Blood of Kala Azar Patients.

Caronia (1913) investigated the amount of complement in the blood of patients infected with *Leishmania infantum*, and found that there was a marked increase in the complemental power of the blood, especially in the late stages of the disease. Varying quantities of serum were mixed with a constant amount of washed ox red cells and anti-bovine hæmolysin, and incubated at 37° C. for one hour. The smallest quantity of serum necessary to produce complete hæmolysis was thus obtained. This author found that in sixteen patients the amount of serum necessary to produce complete hæmolysis varied between 0·07 c.c. and 0·03 c.c., with an average of about 0·04 c.c., whilst seven controls averaged 0·12 c.c.

We have made a few experiments to determine whether there was any such increase in the amount of complement in patients suffering from Chinese Kala Azar, and with this object, varying quantities of serum were mixed with a constant amount of sensitized sheep red cells and incubated at 37° C. for one hour. In six Kala Azar patients the amount varied between 0·04 and 0·1 c.c. of serum necessary to produce hæmolysis, whilst eight controls gave figures varying between 0·03 and 0·12 c.c. Our results, therefore, seem to indicate that in Chinese Kala Azar there is no marked increase in the complemental power of the serum.

Complement Fixation.

Di Cristina and Caronia (1913) were the first to test the complement fixation in the case of infantile Kala Azar and obtained a partial or definitely positive result in 13 cases out of 88. Moses (1919) obtained much better results in the case of South American Leishmaniasis for, employing a flagellate antigen, 80 per cent. were found to give a positive reaction.

The results of our experiments suggest that with a suitable antigen this test may be of value in the diagnosis of Kala Azar. As antigen, we employed the spleen of hamsters infected with the disease, for in this animal the number of parasites is far in excess of that in the human being. The infected spleen was triturated with about 5 c.c. of normal saline solution, and after the larger particles had been removed by straining through muslin, 1 per cent. glycerine and ½ per cent. phenol were added to the suspension. This antigen was then tested to see how much complement it absorbed. 0·8 c.c. of the suspension was mixed with 0·2 c.c. of inactivated normal human serum and varying quantities of 5 per cent. guinea-pig serum for complement, and incubated at 37° C. for one hour. One c.c. of 5 per cent. sensitized sheep red cells was then added to each tube and the mixture again heated to 37° C. for one hour. The tube in which hæmolysis was complete indicated the amount of complement absorbed by the antigen and normal inactivated serum. 0·2 c.c. of the serum to be tested, after being inactivated, was then mixed with 0·1 c.c. of the antigen in a number of tubes, to which were added guinea-pig complement sufficient to produce complete hæmolysis with normal serum and antigen, and then incubated at 37° C. for one hour. Subsequently, 1 c.c. of 5 per cent. sensitized sheep red cells were added to each tube and the mixtures again incubated for one hour.

We have tested this method on 24 cases of Kala Azar, diagnosed by liver puncture, and in 19 cases obtained a well-marked reaction. In five cases there was only a partial reaction, but even here the hæmolysis was less than in the controls.

The blood of 54 patients suffering from various other diseases has also been tested to see whether the reaction is specific to Kala Azar. In 44 hæmolysis was complete; in four it was incomplete, whilst six gave a definite reaction, hæmolysis being absent. Five of these cases were syphilitics and had given strongly positive Wassermann reactions, whilst the blood of the other case contained anti-complement.

In addition to the above, the blood was tested of three Kala Azar patients who had been successfully treated by injections of tartar emetic, and in each case the result was negative.

The results of our experiments suggest that by using a suitable antigen, such as a suspension of the spleen of a heavily infected animal, the complement fixation test may be of value in the diagnosis of Kala Azar, but it should be used in conjunction with the Wassermann test, as certain cases of syphilis, and also blood containing anti-complement, may give a positive reaction.

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Reports from the Royal Society's Kala Azar Commission in China.

No. 2.—Experiments Bearing on the Susceptibility of the Striped Hamster (Cricetulus griseus) *to* Leishmania *of Chinese Kala Azar.*

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The experimental study of Kala Azar has been greatly facilitated by the introduction of the use of the Chinese striped hamster (*C. griseus*) as a laboratory animal. Smyly and Young (1924) were the first to demonstrate the marked susceptibility of this animal to infection with *Leishmania*, and subsequently, in collaboration with Brown (Young, Smyly and Brown, 1926), they confirmed and extended their original observations. Utilizing large numbers of hamsters, they were able to show that the intraperitoneal route is the most certain method of infecting this rodent. Out of a total of 182 hamsters inoculated in this way, 129 were positive after intervals varying from 10 to 347 days; and they noted that the parasites originally inoculated into the peritoneum could persist there for about a month after they were introduced. Two hamsters were infected by intrapleural injection, and both became infected, one after 15 days, and the other after an interval of 67 days. The infection in each case was detected by liver puncture.

With regard to subcutaneous injection of the parasites into hamsters, these authors only succeeded in infecting three hamsters out of a total of 14. In connection with all their experiments it should be noted that their animals were invariably inoculated with relatively large quantities of liver and splenic emulsion, rich in parasites. It is important to note that in spite of the fact that large numbers of parasites were usually present in the spleen and liver, and the former organ was greatly enlarged, the animals seemed to remain in good health for long periods; although no tendency to spontaneous recovery was ever observed.

Young and Liu (1925) have recently shown that the giant hamster, *Cricetulus triton*, appears to be nearly, if not quite, as susceptible as its smaller ally, *C. griseus*. This larger rodent similarly showed no tendency to spontaneous recovery.

EXPERIMENTS WITH THE NON-FLAGELLATE FORM.

As the result of a few inoculation experiments, we are able to confirm the foregoing observations on the susceptibility of *C. griseus* to *Leishmania.* But whereas the above authors had a considerable number of negative results, we seem to have had a larger percentage of successes. Out of 36 hamsters inoculated intraperitoneally with material from infected animals, 35 became infected. One animal died early on in the experiment, and as its organs were too far decomposed before it was examined, it is regarded as a negative result. It should be noted that the 36 hamsters were inoculated with large numbers of parasites as in the case of the experiments recorded by the above authors.

We next inoculated a number of hamsters from material obtained by liver puncture from cases of Kala Azar in the wards of the hospital of the Shantung Christian University Medical School, in order to note any difference in virulence, degree of infection, etc. In each case, the material used contained only a very small number of parasites.

Experiment 1.—In this experiment the material was obtained by liver puncture from a case of Kala Azar which proved fatal later; parasites had earlier been found in films from the finger blood of the patient. The contents of the needle used in puncturing the liver of the case were emulsified in sterile saline, and inoculated intraperitoneally into three hamsters. From microscopic examination of films of the material it was estimated that not more than 20 parasites were introduced into each hamster. All three became infected, two giving a positive liver puncture 52 days after inoculation, and in the third parasites were detected in the spleen and liver at the post-mortem examination later. It should be noted, however, that in these three hamsters the course of the infection differed from that observed in our other experiments. The first animal was killed six months after inoculation, and very few parasites could be detected in any of the organs. Many of these parasites were obviously degenerating, the micro-nucleus appearing swollen, and the protoplasm of the parasite staining badly. There was no enlargement of the spleen. The second animal is still alive at the time of writing (April 24, 1926), and shows no increase in the number of parasites as determined by liver puncture. After three negative liver punctures, the third animal was killed 211 days after inoculation. The spleen was very slightly enlarged, but otherwise the animal appeared to be in good health. Smears from the spleen and liver showed the presence of extremely few parasites, and many badly staining single forms suggestive of degeneration.

Experiment 2.—Two hamsters were inoculated intraperitoneally with an emulsion of spleen pulp in Locke's solution from a fatal case of Kala Azar 10 hours after death. This case had received three weeks intensive treatment with Stibamine Glucoside, and films made from the spleen after death were negative. Both animals showed parasites in large numbers in smears made from liver puncture. In both cases a large quantity of spleen pulp was used for inoculation. Attention may be drawn to the viability of the parasite in the human body 10 hours after death.

Experiment 3.—A hamster was inoculated intraperitoneally with material obtained by liver puncture of a boy suffering from Kala Azar, believed to be of six years' duration. The animal proved to be positive by liver puncture three months later. Many similar experiments are in progress, and will be reported later.

In all the animals which have been examined up to the present, in which a small number of parasites have been inoculated, no obvious enlargement of the spleen has been noted, in marked contrast with the usual appearance of this organ in hamsters infected by massive doses, when the spleen is often very much enlarged, sometimes stretching across the abdominal cavity to the right side.

Inoculation of Hamsters by Routes other than the Intraperitoneal.

I. *Percutaneous.*—In each case the skin of the thorax of a hamster was shaved by means of a razor, and a drop of spleen emulsion rubbed on to the excoriated surface and allowed to dry. Owing to the site of the inoculation it was difficult for the animals to lick the shaved surface, and this was not observed. Nine hamsters were inoculated in this way. They were examined by liver puncture after an interval of 10 weeks and all found to be negative. Four of these animals were then inoculated intraperitoneally with a splenic emulsion of the parasite from an infected hamster. They have since become infected, showing that the previous experiment failed to produce any immunity. The remaining five were still negative 3½ months after inoculation. Although in each case the site of the infection was carefully examined, no local lesion of any kind developed, such as has been described by Row (1912) in the case of a monkey inoculated in a similar way with the parasite of Indian Kala Azar.

II. *Subcutaneous Inoculation.*—Ten hamsters were inoculated subcutaneously, each with 0·3 c.c. of a rich suspension of the parasite in saline. Here again they were all negative to liver puncture after an interval of 10 weeks. Four were then given an intraperitoneal inoculation and became infected. The remaining five were killed after 3½ months, and of these four were negative and one positive. These results are in accord with those of Young, Smyly and Brown, who, as already noted, only succeeded in infecting 3 out of 14 hamsters inoculated subcutaneously. It is evident that the percutaneous and subcutaneous routes are not nearly so certain a method of infecting this animal as the intraperitoneal.

EXPERIMENTS WITH FLAGELLATES.

Assuming that the flagellate form of the parasite is the infective stage in nature, it seemed important to try and discover whether the hamster could be infected with this phase of the parasite. Up to the present no animal inoculation experiments have been recorded with this stage of the parasite of the Chinese form of the disease.

Intraperitoneal Inoculation. Experiment 1.—Five hamsters were inoculated intraperitoneally with 0·2 c.c. of a 12-day culture in N.N.N. medium containing about one parasite to a field. All were negative to liver puncture for four months, when four were killed and examined. Two of these were negative microscopically, and from the organs of one cultures were made which also proved to be negative. Two other animals became infected. One showed a very scanty infection, with no enlargement of the spleen, and only a single parasite was found, in films from the liver, after a long search. The other animal also showed no microscopic signs of infection, but large numbers of parasites were found in smears from the spleen and bone marrow, and rather fewer in those made from the liver. The fifth animal was killed about six months after inoculation and proved to be negative.

Experiment 2.—In this series of experiments we used a 14-day-old culture, rich in flagellates, which had been grown on N.N.N. medium from a fatal case of Kala Azar, and the following routes of infection were employed:—

(*a*) *Percutaneous.*—In each case a drop of the culture was rubbed on to the shaved and excoriated skin of the thorax of a hamster and left to dry. Ten hamsters were inoculated in this way. All remained negative to liver puncture for about 2½ months, but during this time two died, and examination of their organs proved negative, although one had a slightly enlarged spleen. At the end of 2½ months, three of the animals were inoculated intraperitoneally with 0·2 c.c. of spleen and liver emulsion of another heavily infected hamster; all three became infected after a short interval. The remaining five were killed about four months after the inoculation, and smears made from the spleen and liver were carefully examined. One of the animals showed a heavy infection of the spleen and liver, especially of the former, but no enlargement of that organ was noted. The remaining four hamsters were negative.

(*b*) *Subcutaneous.*—Nine hamsters were inoculated subcutaneously with 0·2 c.c. of the same culture of the parasite. One of these died two months later, and on examination was negative. The remainder were negative to liver puncture about 2½ months after inoculation, when three were inoculated intraperitoneally with spleen emulsion from a heavily infected hamster. The remaining five were killed 3½ months after inoculation, and of these four were negative, but the fifth showed a very scanty infection of the spleen, which was normal in size and appearance. No parasites could be found in smears from the liver. The hamsters which were inoculated intraperitoneally became infected after short incubation periods.

(*c*) *Intraperitoneal.*—Nine hamsters were inoculated intraperitoneally with 0·2 c.c. of the culture. Of these, all except one showed a heavy infection of spleen and liver after periods varying from 2–3 months after inoculation. Two months after inoculation most of them showed parasites in smears made from liver puncture.

The above experiments demonstrate that the Chinese striped hamster (*C. griseus*) is very susceptible to infection with flagellates by the intraperitoneal route, and that it may also be infected by the percutaneous and subcutaneous routes.

Conclusions.—Summarizing the results of the above experiments it will be noted that the Chinese striped hamster is markedly susceptible to intraperitoneal inoculation, using both the non-flagellate and flagellate stages of the parasite. This rodent is, therefore, eminently suitable for transmission experiments. In addition, it lives well in captivity, and remains infected for long periods without showing any obvious signs of ill-health. When very small numbers of parasites are inoculated by any of the routes mentioned, and especially when the flagellate stage is employed, the resulting infection may be slight, so that it may quite easily be missed ; and even when considerable numbers are present, there is commonly no enlargement of the spleen. On the other hand, with one doubtful exception, intraperitoneal inoculation with large numbers of parasites always resulted in a heavy infection, and great enlargement of the spleen, and in these cases the parasite could be recovered from the peripheral blood.

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Reports from the Royal Society's Kala Azar Commission in China.

No. 3.—Notes on Kala Azar in Shantung.

By Major W. S. Patton, I.M.S. (Retd.), and Edward Hindle, M.A. (Camb.), Members Kala Azar Commission of the Royal Society (N. China).

(Received May 26, 1926.)

Through the kindness of the authorities of the hospital of the Shantung Christian University Medical School, we have been able to examine the records of the hospital for the past six years, from July, 1920, up to the present date, and to collect together the data relating to 301 patients suffering from Kala Azar, admitted to the hospital during this period. The information contained in these records is summarized under the following heads:—

(1) *Sex Incidence.*—Analysis of the records shows that, up to date, 270 males and 31 females were admitted into the hospital. These figures, however, cannot be considered to represent the truth regarding the sex incidence of the disease, as female patients are much less likely to be brought to hospital. But from local information from residents who have some knowledge of the disease, we gather that the disease is distinctly commoner among males.

(2) *Age Incidence.*—Young, in his paper on "Kala Azar in China" (1923), summarized the available information regarding the age incidence of the disease, based on the analysis of 762 cases from various sources. His results are as follows:—Ages 1–10, 31·5 per cent.; 11–20, 40·1 per cent.; 21–30, 18·4 per cent.; 31–40, 7·9 per cent.; 41–50, 1·8 per cent.; 51–60, 0·3 per cent.

The Shantung Christian University Hospital records give the following figures:—Ages 1–10, 32·2 per cent.; 11–20, 32·5 per cent.; 21–30, 22·8 per cent.; 31–40, 8·4 per cent.; 41–50, 3 per cent.; 51–60, one isolated case. All these records indicate that the disease is one of the earlier decades of life, but although the figures suggest that the second decade possesses the highest incidence, it is unlikely that this represents the actual facts. Out of the 301 cases admitted to hospital, only three were of children under 5 years of age. The difficulties attending the bringing of children under this age, often from long distances, to hospital, are so great that it is exceptional for Chinese country people to bring such small children for treatment. As far as can be ascertained, very young children are just as susceptible as older ones. Therefore it can be assumed that the disease in China is most prevalent in the first decade, and gradually diminishes, until it is very rare after 40 years.

(3) *Seasonal Incidence.*—In view of the interesting results obtained by McCombie Young in India, the cases have been arranged according to the months of admission to the hospital, in order to determine whether there is any evidence of a seasonal incidence of the disease in China. The figures in the second column were compiled from dates of onset of the disease as given by the patients themselves.

Months.	*No. of Cases.*	*Month of Onset.*
January	6	13
February	27	12
March	41	11
April	33	13
May	26	10
June	19	16
July	23	8
August	20	11
September	22	11
October	32	11
November	25	13
December	17	7
	301	

These figures give no evidence of any seasonal incidence bearing on the etiology of the disease, but rather depend on the habits and occupations of the people. During December and January the cold weather deters people from coming to hospital; and no patient likes to be in hospital during the Chinese New Year, which usually falls about the beginning of February. After this festival there is a marked increase in the number of patients, as there is not much work to be done on the land during the latter part of February and during March. The differences in the number of admissions for the remaining months are negligible. The figures in the second column relating to the months of onset of the disease, as stated by the patients, also give no evidence of any seasonal incidence.

(4) *Duration.*—It is difficult to fix any duration of the disease, but the following figures, based on the patients' own statements, seem to indicate that Kala Azar in China is of rather longer duration than the Indian form. It should be emphasized that these figures only represent the length of time that the patient felt any symptoms of the disease before coming to hospital. But the disease is very well known among the country people of Shantung, the enlarged spleen being indicated by the local name "Chang-pih," meaning "lump in the abdomen," and therefore a certain amount of reliance may be placed on the statements concerning the duration of symptoms.

Duration of Disease.	No. of Cases.
Less than 6 months	32
6 months to 1 year	51
1 to 2 years	75
2 to 3 years	50
3 to 4 years	17
4 to 5 years	10
More than 5 years	9
	244

Mackie gives the following figures for 250 cases observed in Assam :—

Duration of Disease.	No. of Cases.
Less than 6 months	65
6 months to 1 year	99
1 year to 18 months	27
18 months to 2 years	33
2 to 3 years	14
More than 3 years	12

A comparison of the two tables shows that the mean duration of the disease in China is 1 to 2 years, whilst in Assam it is 6 months to 1 year. Moreover, in the former there are many more cases of longer duration than 2 years. Some of the patients have given statements indicating a duration of symptoms up to as long as 15 years, and in view of the chronic nature of the infection in the hamster (*C. griseus*), there is no reason to doubt that in certain cases the disease may be of very long duration.

(5) *Epidemiology.*—A study of the hospital records and also local information clearly indicates that in Shantung the disease is especially prevalent in country villages and not in the towns. Seventy-five per cent. of the patients were farmers, and most of the remaining 25 per cent. were either small village shopkeepers or school children. Although widespread, the disease is by no means evenly distributed throughout the province, certain districts being more heavily infected than others. Moreover, one village may be infected and neighbouring villages free from infection. The disease seems generally to be of an endemic nature with occasional epidemics, in this respect resembling Indian Kala Azar.

Through the kind assistance of Dr. C. H. Han and Mr. Yiu of the Shantung Christian University, we have made a survey of one village, Chen Li Chia, in the north-east of the province, in which an epidemic occurred about 15 years ago. The distribution of the cases is indicated in the attached plan of the village. Out of 224 houses, cases of Kala Azar had occurred in 50 of them, irrespective of the social conditions of the occupants. Thirty-four houses had only one case; 7 houses had two cases; 5 had three; 2 had four; and 2 houses each had records of five cases, making a total of 81 cases.

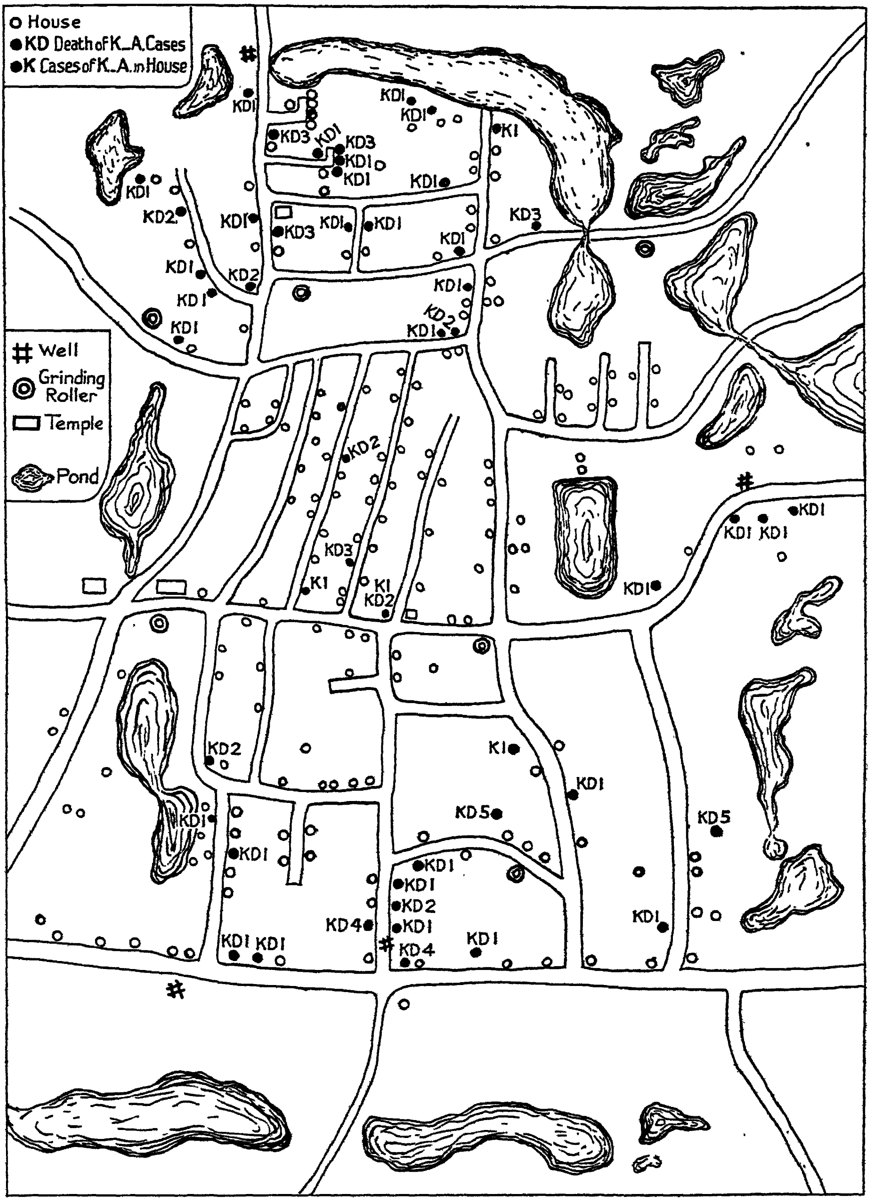
It is of interest to note that the local inhabitants state that the disease first came to their village about twenty years ago and was especially prevalent between 1910 and 1915. It was so dreaded that people of the neighbouring villages would not buy farm produce or employ labour that came from the infected village. At that time most of the deaths occurred in people about the age of twenty, but at present it is restricted to infants, and at the time of our survey the only cases existing were one of 6 years and three of 12 years old.

The villagers stated that the disease came from the west and is now spreading towards the east of the plain, and an epidemic of Kala Azar was said to be in progress in a village about 20 miles east of Chen Li Chia. Unfortunately, owing to the prevalence of bandits in that region, it was impossible to verify the latter statement by a personal investigation.

Forty-three out of the 301 patients in the local hospital gave a history of other cases of Kala Azar occurring in their families, and the occurrence of more than one case in the same house is also shown in the records of Chen Li Chia. There is no evidence, however, of any site infection, such as was found by Patton (1907) at Madras, for in the majority of cases only one member of the family was attacked by the disease. But almost every patient gave a record of other cases occurring in his village or the surrounding neighbourhood.

(6) *Incubation Period.*—In view of the uncertainty of the incubation period.

of the disease, the observations on the following case are of interest, since they indicate a minimum incubation period of 10 months.



The patient, a female student in the Medical School, aged 22 years, complained of lassitude and loss of appetite in April, 1925, and between March and May began to lose weight, as shown in the records of the students' weights, which are taken every two months. The condition was not diagnosed until 21st October, 1925, when the spleen was found to be slightly enlarged, and liver puncture revealed the presence of *Leishmania.*

Previous to this date the patient had lived in the students' quarters at the Shantung Christian University, Tsinan, since June, 1924, when she spent 15 days in the compound of the Shadyside Hospital at Wei-hsien. All the available evidence shows that Kala Azar does not occur in Tsinan, and therefore the patient must have become infected before coming into residence in June, 1924. The district around Wei-hsien, on the other hand, is a well-known centre of infection, and many cases of the disease were under treatment at the hospital whilst she was staying in the Mission compound. Previous to visiting Wei-hsien, the patient had spent two years in Peking, and, moreover, her home, Ching-chow-fu, is an infected neighbourhood, therefore infection might have been acquired previous to the visit to Wei-hsien. It seems unlikely, however, that the disease should have remained dormant for such a long period, and therefore we consider it highly probable that the patient became infected at Wei-hsien. This gives an incubation period of 10 months before any symptoms whatever developed, and even after 16 months the spleen was only very slightly enlarged.

The patient was treated with intravenous injections of tartar emetic, and made an uninterrupted recovery.

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Reports from the Royal Society's Kala Azar Commission in China.

No. 4.—*Resistance of* Leishmania *Cultures to Cold.*

By Edward Hindle, M.A. (Camb.), and Major W. S. Patton, I.M.S. (Retd.),
Members Kala Azar Commission of the Royal Society (N. China).

(Received May 25, 1926.)

On the assumption that *Leishmania* is able to persist outside the vertebrate host, it is evident that in North China the parasite must be capable of withstanding exposure to extreme cold, for during the winter the temperature in Shantung Province often reaches — 20° C. If the parasite is able to persist throughout the winter in the body of some insect, the flagellate stage should not be killed by exposure to low temperatures.

So far as we are aware, no observations have hitherto been recorded on the resistance of flagellates to cold, and, therefore, we examined a number of hibernating insects with a view to finding out whether natural flagellates could persist during the winter. Active flagellates were found in the alimentary tract of hibernating females of *Culex fatigans*, and in a Pentatomid bug caught in January, and also in the same species of bug in March, after it had just emerged from hibernating during the winter, thus showing that natural flagellates of these insects are not affected by cold. In addition, experiments were made with cultures of *Leishmania*, in order to determine whether the flagellate stage of this parasite was equally resistant to cold.

Experiment 1.—Four actively growing 12-day cultures of *Leishmania* on Wenyon's N.N.N. medium were placed overnight outside the window of the laboratory ; during the night the temperature fell to — 10° C. The next day one of the cultures was examined, after being two hours at room temperature, and found to contain active flagellates. All the cultures were then kept outside for six days, during which the night temperature varied between — 6° C. and — 10° C., and the day temperature rarely rose above freezing point. The cultures were then kept at 20° C. for three hours and all contained active flagellates.

Experiment 2.—Six cultures containing numerous active flagellates were placed outside, against a recording thermometer in the grounds of the University. The night temperatures during the succeeding 14 days varied from — 7° C. to — 12° C., and the maximum day temperature only once rose as high as 6° C.

One of the cultures was examined after three days and, after one hour at 20° C., contained active flagellates ; it was returned outside and re-examined after an additional five days, and, after four hours at 20° C., contained a few motile flagellates and also many non-motile forms. It was kept outside, and re-examined two days later, when it still contained a few active flagellates, but after a further period of six days was negative. Two other cultures were removed after 10 days' exposure to cold and both contained many active flagellates after three hours at 20° C. The remaining cultures were negative after 18 days.

Experiment 3.—Five actively growing cultures, four on Wenyon's N.N.N. and one on Noguchi and Lindenburg's medium, were placed in the ice chest. Six days later two of the cultures were removed to a temperature of 20° C. Both of these cultures were negative after one day, but, after six days at 20° C., one of them (on Noguchi and Lindenburg's medium) contained a few flagellates, whilst the other, on Wenyon's N.N.N., was still negative. These two cultures were then kept at room temperature for another 14 days, when the latter was found to contain numerous flagellates, whilst the other had become negative. The remaining three cultures were removed from the ice chest after 20 days and kept at room temperature, but all remained negative.

The results of these experiments clearly show that the flagellate stage of *Leishmania* is able to withstand intermittent exposure to temperatures of — 12° C. for a period of at least 10 days. Moreover, the flagellate stage, as such, is evidently able to withstand freezing solid, for within one hour of being thawed active flagellates could be observed in the cultures.

As the parasite can persist in this manner when exposed to cold under the abnormal conditions of culture media, where changes in the hydrogen-ion concentration are taking place, it is reasonable to assume that under natural conditions, such as in the alimentary tract of some hibernating insect, the flagellate might be able to persist throughout the winter.

Reports from the Royal Society's Kala Azar Commission in China.

No. 5.—Notes on Three New Parasites of the Striped Hamster (Cricetulus griseus).

By Major W. S. Patton, I.M.S. (Retd.), and Edward Hindle, M.A. (Camb.), Members Kala Azar Commission of the Royal Society (N. China).

(Received May 26, 1926.)

In the course of our experiments various natural parasites of the hamster have been observed, in addition to the usual intestinal fauna, and we append a brief description of three of these, *Trypanosoma cricetuli*, n.sp., *Grahamella cricetuli*, n.sp., and *Sarcocystis cricetuli*, n.sp.

On the whole, this animal is remarkably free from ectoparasites, for during the examination of more than 800 hamsters we have only found them on two occasions. *Hœmatopinus* sp. was found in very large numbers on one emaciated individual, but, although it doubtless occurs on a small percentage of these animals, we have never succeeded in finding it again. Young and Hertig (1925) attempted to transmit *Leishmania* by means of this louse, but failed to obtain any evidence either of transmission or of development of the parasite in the alimentary canal of the insect. These results, combined with the rarity of this ectoparasite, indicate that in using the hamster for experiments on the transmission of *Leishmania*, *Hœmatopinus* may be neglected as a complicating factor.

In addition, a single specimen of *Ceratophyllus* was caught on one individual, and one seen, but not caught, on another. It is, therefore, rather surprising that about one-third of the hamsters should contain blood parasites which are most probably transmitted by their agency.

Trypanosoma cricetuli, n.sp.

This parasite was found in the blood of about one-third of the hamsters examined, and as a comparatively short time was spent on the examination of the blood of each animal, probably a larger proportion of them are infected. Occasionally the trypanosome was found in considerable numbers, ten or more in each field of the microscope. No pathogenic symptoms were observed in any of the infected hamsters, even in those containing large numbers of trypanosomes, and one may reasonably assume that, as in the case of most natural infections, a balance has been established between the parasite and its host.

The *living trypanosome* exhibits the usual wriggling movements of these parasites, and in addition shows the rapid translatory activity of *T. lewisi.* Its movements, however, are more flexible than those of the rat trypanosome, and the undulating membrane is more distinct.

In *stained preparations* the trypanosome is seen to be monomorphic. Its shape is indicated in fig. 1, from which it will be seen that the body of the animal is prolonged into a pointed posterior extremity. The flagellum with the undulating membrane is of average dimensions, and anteriorly is free for a comparatively short portion of its length. The cytoplasm is almost free from granules. The kinetonucleus is large, elongated oval in shape, and arranged transversely to the length of the body, some distance from the posterior extremity. The trophonucleus is situated about the middle of the length of the body of the trypanosome.

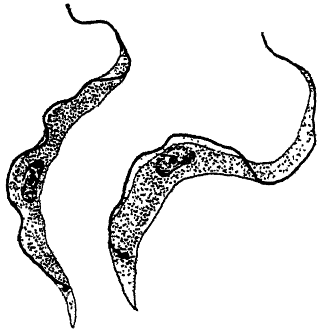
FIG. 1.—*Trypanosoma criceluli.*

The dimensions of the trypanosome show very little variation, probably owing to the fact that most of the specimens observed had reached the non-reproductive phase. The average dimensions are as follows :—

Total length of trypanosome from posterior extremity to tip of flagellum, 28μ ; width of body at trophonucleus 2μ ; distance from posterior extremity to kinetonucleus, 3·5μ ; distance from kinetonucleus to centre of trophonucleus, 8·5μ ; length of free flagellum varying between 3μ and 7μ.

This trypanosome occurs in the blood of the striped hamster, *Cricetulus griseus,* and in view of its host we propose for it the name *Trypanosoma cricetuli.*

Grahamella cricetuli, n. sp.

On various occasions we have observed parasites belonging to the genus *Grahamella* in the red cells of hamsters, *Cricetulus griseus,* caught at Wei-hsien, Shantung Province, China, some of which were also infected with trypanosomes.

The parasite was comparatively scarce in numbers, at most one red cell being parasitised in every forty fields of the microscope.

In stained preparations the parasite has the appearance of rounded, comma-shaped, or bacilliform bodies, inside the red cells, some of which may contain very large numbers of these organisms. If Romanowsky's stain is used, the parasite stains intensely, and in most of them one or more chromatic granules can be seen. Some of the organisms, however, stain a uniform blue colour, and give no evidence of the presence of any chromatin. Bipolar forms suggestive of transverse division may be observed, and therefore the parasite seems to fall into the genus *Grahamella*, Brumpt, distinguished from *Theileria* by the absence of quadruple division forms. We propose the specific name *cricetuli*, in view of the host, *Cricetulus griseus*.

Sarcocystis cricetuli, n. sp.

Sarcosporidia are so widely distributed in nature that it is not surprising that examples of *Cricetulus griseus* were also found infected with these organisms. The parasite was found in one out of 157 hamsters obtained from Peking, and in 7 out of 155 hamsters caught at Wei-hsien, Shantung Province. The cysts are confined to the subcutaneous muscles of the back and pharynx, and are comparatively few in number, not more than 20 having been observed in any one animal. If the hair is removed the cysts can be seen through the semi-transparent skin of the living hamster.

The average size of the cysts is 1·5 mm. by 0·2 mm.; they are of the usual elongated oval shape. When the animal is skinned, the cysts generally adhere to the inner surface of the skin, where they form conspicuous white spots.

The cysts all contained large numbers of spores, apparently at a similar stage of development. Each spore is elongated and slightly curved, with one end rounded and the other pointed. In stained preparations the large nucleus is observed lying near the rounded extremity. Occasionally, in specimens stained with iron hæmatoxylin, at the opposite end is either a vacuole or a clump of granules, and the cytoplasm is also sometimes filled with small granules. The size of the spores is from 8 to 10 μ in length by 2 μ in width.

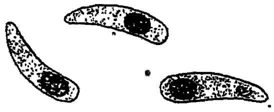

FIG. 2.—Spores of *Sarcocystis cricetuli*.

Twelve hamsters were fed with a suspension of spores in saline, from cysts of a freshly killed animal. Three of these died within the next 14 days and were negative. Another died after 7 weeks, and a few small cysts containing spores were found in the subcutaneous muscles of the back. The remaining eight hamsters were killed four months after the experiment, and were all negative. These results suggest that the parasite is not transmitted by swallowing the spores, as in the case of *Sarcocystis muris*, for the single positive animal had probably acquired infection previous to the experiment.

As this parasite differs from other described species of *Sarcocystis*, both in its host and in the site chosen for the development of the cysts, we consider it to be a new species, for which the name *S. cricetuli* is proposed.

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The Action of X-Rays on Colpidium Colpoda.

By J. A. Crowther, M.A., Sc.D., F.Inst.P., Professor of Physics in the University of Reading.

(Communicated by Sir William Hardy, F.R.S.—Received July 1, 1926.)

The action of X-rays on living tissues is a subject not only of much practical importance, but also of considerable theoretical interest. While there is in existence an immense accumulation of observations on the subject of the most varied kind, comparatively little of it has that numerical basis which is necessary for the foundation of any physical theory of the action. Some little time ago I ventured to suggest* that some interesting results obtained by Strangeways and Oakley† on the action of X-rays on tissue cells could be accounted for by the well-known discontinuous nature of the absorption of X-rays by matter, assuming that the structures in the cell which were vitally affected by the rays were sufficiently small for this effect to come into operation. Calculation showed that the size of structure required was not unduly small in comparison with that of structures known to exist in tissue cells.

These results were explained on the assumption that a single ionisation by the X-rays of the particle concerned would be sufficient to produce the observed biological change. An extension of the theory to cover cases in which a succession of such hits is required presents no great difficulty, and the relationships obtained in this way between survival and dosage are found to agree, generally, with the results of other experiments, as, for example, those of Dr. F. C. Wood on cancer cells. I had been hoping that results would be published which would enable the theory to be tested numerically, but, so far as I am aware, such numerical results have not appeared. The present experiments were commenced in the hope of obtaining such numerical data.

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* Crowther, 'Proc. Roy. Soc.,' B, vol. 96, p. 207 (1924).

† Strangeways and Oakley, 'Roy. Soc. Proc.,' B, vol. 95, p. 373 (1923).

The choice of an organism for the experiments was governed by several factors. In the first place, it was desirable that it should be as simple as possible. The effects produced by the irradiation of certain of the tissues of a complex organism may be largely secondary in their origin, and the problem is sufficiently complicated even when reduced to its simplest terms. The effect produced by the radiation on the organism must be one which is easily recognisable, and which lends itself to numerical estimation. In the present experiments it was also necessary to have an organism which was not too susceptible to slight changes in environment, which would not demand, in its handling, a too difficult or specialized technique, and which would sustain without damage the treatment it would be likely to experience in the course of the experiments.

It seemed possible that one of the Protozoa might meet these requirements. I am indebted to Mr. D. Ward Cutler for suggesting *Colpidium Colpoda*, and for his further kindness in supplying me with a stock of that organism. This stock had been raised originally from a single individual. It was grown in Glycerine-Ammonium Phosphate solution, and was infected with a single species of bacteria. The production of the necessary sub-cultures from the original stock was very kindly undertaken for me by Mr. W. Buddin. This particular strain has been extensively studied both by Mr. Cutler* and by Dr. Peters, and its behaviour under various circumstances is thus well known.

One possible drawback to the use of this otherwise very suitable material was the fact that previous observers had failed to demonstrate any action of X-rays on any protozoan, in spite of prolonged exposures. Since this work was commenced Hance and Clark,† using very large secondary currents with a broad focus Coolidge tube, have observed small irregular changes in the rate of division of certain paramecia, but even with the large doses employed by them no lethal effect was observed. Fortunately I had at my disposal (owing to the generosity of the Government Grants Committee of the Royal Society) the means of producing a very powerful beam of X-rays, and it was thought that this difficulty might not be insuperable. This proved to be the case.

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* Cutler and Connys, 'Biochem. Journ.,' vol. 17, p. 174 (1923); vol. 18, p. 905 (1924).

† 'Journ. of Exp. Med.,' vol. 43, p. 61 (1926).

Experimental Arrangements.

The source of X-rays was an experimental tube of the type devised by Dr. Shearer, exhausted by a mercury condensation pump. The tube was permanently connected to the pump, and the vacuum was regulated to any desired value by means of a needle valve connecting the tube to the outer air. A large induction coil with a mercury interrupter supplied the high-tension current for the tube. Throughout the experiments the tube was furnished with a molybdenum anticathode, and the tube was run at a P.D. of about 50,000 volts. Under these circumstances the radiation should consist principally of the K radiation of Molybdenum. As no X-ray spectrometer was available this was not directly verified, but the coefficient of absorption of the radiation in aluminium $\left(\frac{\lambda}{\rho}=3\cdot 2\right)$ was consistent with this assumption. It is assumed, therefore, that the radiation used was mainly Molybdenum K radiation, the principal lines of which have wave-lengths 0·625 and 0·71 Å.U.

The intensity of the radiation from the tube was measured by an ionisation chamber of a simple air-gap type. The rays were admitted to the chamber through an aperture, 1 cm. × 0·5 cm. in a lead plate, and passed through the chamber without falling either on the electrodes or the walls, passing out through a suitable aperture at the far end. The path of the rays between the electrodes was 2 cm. A subdivided microfarad condenser was connected to the insulated plate of the ionisation chamber, and the capacity was adjusted so that the system charged up to a potential of approximately 1 volt in the course of one complete exposure. The charge passing across the ionisation chamber, measured in microcoulombs, affords a measure of the dosage given.

To make an exposure a small drop of the culture containing a suitable number of the protozoa was placed in the centre of a counting chamber, 0·1 mm. deep, and covered with a glass cover-slip, 0·13 mm. thick. A ring of small drops previously placed in a groove surrounding the central part of the counting chamber helped to prevent evaporation from the main drop. The drop, thus mounted, was centred below the window of the X-ray tube by a simple geometrical clamp. The distance of the drop from the window of the tube was 0·84 cm. and from the focal spot of the anticathode 2·50 cm. The distancce of the window of the ionisation chamber from the focal spot was 7·30 cm. The dose given to the slide was thus 8·5 times that measured by the ionisation chamber. A charge of 1 microcoulomb in the ionisation chamber is thus equivalent to a dose of 25,500 *e* on the specimen, where *e* represents Friedrich's unit of dosage. With the apparatus described a dose of this magnitude could be applied in from six to seven minutes.

Cutler had already demonstrated that the Colpidia will live for very considerable periods in drops prepared in this way. As an additional check, however, a control slide was always prepared. The two slides were then left side by side at the ordinary laboratory temperature for an hour, after which one of them was exposed to the radiation. The number of Colpidia in each slide was counted under a microscope and their appearance and condition recorded. In the large majority of cases no change was observed in the control, even after a period of several days. In a few slides, however, deaths occurred within the first hour or so after preparation. The experiment with the corresponding test slide was then abandoned.

The maximum number of Colpidia which could be counted with certainty in one drop was not greater than thirty. Experiments had, therefore, to be made on a considerable number of drops in order to accumulate the number of observations necessary for a valid numerical estimate of the effect of the rays. The method had, however, the advantage, firstly, that, owing to the very small thickness of the drop, the intensity of the radiation might be regarded as constant throughout, and, secondly, of allowing the effect of the rays on the individuals to be watched more closely. It was felt that these advantages more than compensated for the tediousness of the method.

Immediate Effects of the X-Rays on Colpidium Colpoda.

In the first experiments the test slide was exposed continuously to a constant beam of X-rays, except for the brief intervals necessary for the examination of the slide under the microscope. The rate of exposure, which was kept as constant as possible, was about 4,000 *e* per minute. Under these circumstances a well-marked series of changes was observed in practically every exposure made. *Colpidium Colpoda* is a freely swimming organism, which is normally in almost constant motion in what is practically a series of straight lines, or smooth curves. A very considerable dose of radiation could be applied without producing any visible alteration either in the appearance or the motion of the organisms. With further exposure, however (1 to $1\frac{1}{2}$ microcoulombs in the ionization chamber), the motion was perceptibly accelerated, and at the same time the Colpidia deserted the centre of the drop, and were seen in rapid and excited circulation around its edge. As the excitement reached its maximum the smooth motion gave place to a rapid zig-zag motion. This appears to be the critical point in the experiment, and if the exposure is stopped at this stage (about 2·5 microcoulombs), the majority of the Colpidia will survive. With slightly longer exposure the zig-zagging develops into a swaying motion, and the speed is greatly diminished; and with a still greater dose all progression ceases, and only the swaying motion is maintained.

At about the same time changes in the structure of the organism become apparent. The vacuole becomes greatly distended, and, not infrequently, a bubble of clear substance is extruded at some point on the organism. These drastic changes necessitate, as a rule, an exposure registered by about 3 microcoulombs in the electroscope. After this stage the organism invariably dies, its death being accelerated by increasing the dose. The death of the organism is indicated by its collapse into a spherical form, and by the complete absence of all movement. As far as I am aware this is the first occasion on which a lethal effect of X-rays has been demonstrated on protozoa of this type. The dose required is rather more than 60,000 *e*, and, as we shall show later, this dose must be concentrated into a comparatively brief interval of time.

As the effect, though not unexpected, was novel, some pains were taken to verify that it was a direct effect of the radiation. As the exposure was made in a ruled counting chamber it was easy to verify that no appreciable evaporation had taken place during the exposure. It was thought possible, however, that with the slide so near the X-ray tube, and with so large an intensity of radiation, the temperature of the drop might be raised during the exposure to an uncomfortable degree. Thermocouples of very fine platinum and copper wires were immersed in the drop and its temperature was thus directly measured. The initial rate of rise of temperature was about $\frac{1}{2}$° C. per minute, and the drop reached a maximum temperature about 2° to 3° above that of the room in about five minutes. Subsidiary experiments indicated that a considerable portion of this rise was due to energy derived from the X-rays absorbed, and not to thermal radiation from the walls of the tube, which were, as a matter of fact, very efficiently water-cooled. Experiments made on drops of the culture, mounted in the usual way, and kept in a thermostat at different temperatures, showed that *Colpidium Colpoda* could sustain a temperature of 33° C. for several hours without any effect. Since the exposures to the radiation were made at laboratory temperatures of from 16° C. to 20° C., the possibility of the effect observed being due to temperature seems to be excluded.

Although we have given an estimate of the mean dose required to produce each of the various effects described, it must be understood that all the Colpidia in a given drop do not pass from one stage to the next simultaneously. Thus, some may still be swimming excitedly round the edge of the drop while others are slowly spiralling in one spot, or have even been killed. The moment of collapse is the one most readily identified, and careful counts were made on a large number of drops to determine the percentage of Colpidia which were killed outright by a given dose of radiation. The curve in fig. 1 gives the results of two series of such experiments, and indicates the percentage of Colpidia surviving the given dose. The circles refer to experiments made with cultures six days old, which have just reached their maximum population. The crosses refer to cultures 18 days old which are beginning to deteriorate. Each point represents the mean of observations made on about 200 individuals. Within the limits of experimental error there is no appreciable difference between the two sets, but to avoid any possible source of confusion and uncertainty the majority of the subsequent experiments were made with cultures from six to seven days old. We will return to this curve in a later section of the paper.

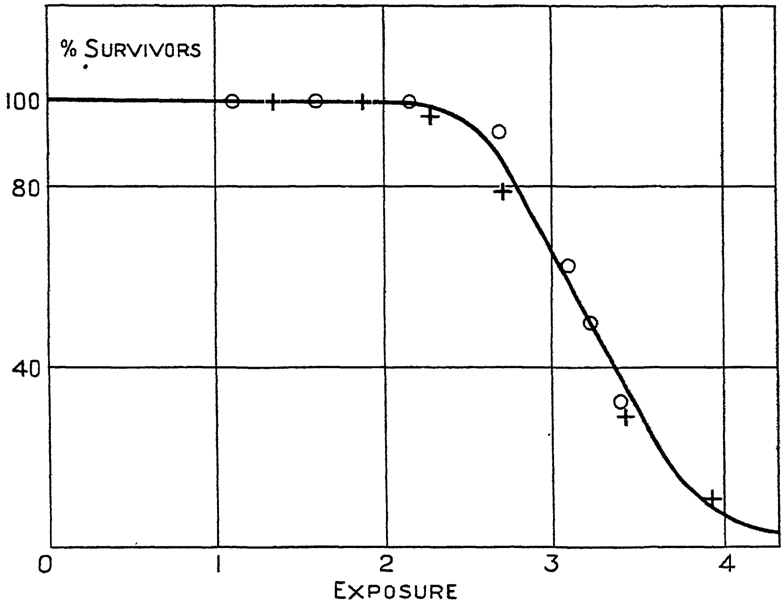

FIG. 1.

Delayed Effects of Irradiation.

The previous section describes the results observed when the culture was irradiated continuously to destruction. Experiments were also made in which the culture was given a definite dose of radiation, less than that which would produce immediate destruction, and then returned to a thermostat (usually at a temperature of 19° C.) for further observation. A "control" was always employed, and was kept under the same conditions as the test slide, except that it was not exposed to the radiation.

When the exposure recorded by the electroscope was less than 1 microcoulomb, no difference could, in general, be detected between the test specimen and the control. If, however, the dose was increased until it produced a marked stimulation in the organisms, a very marked difference was observed between the two slides after an interval of a few hours from exposure. The irradiated Colpidia began to increase markedly in size, until 24 hours after exposure their linear dimensions were 2 to 2½ times greater than those of the control Colpidia. Their appearance was then exactly that of the Colpidia in a culture which has just been sown and is in process of rapid division. The irradiated Colpidia did, in fact, divide, and the number in the drop increased within 36 hours of exposure to nearly twice its original value. No change of any kind was observed in the controls. It seems that a dose of X-rays, rather less than that which is necessary to produce a lethal effect (about 75 per cent. of the lethal dose), produces active division in a culture of Colpidium which has already attained its normal degree of population.

The Colpidia produced under the stimulus of X-rays always contained a proportion of abnormal individuals. These abnormal individuals were considerably smaller than the normal Colpidia, and they were oval in shape without any trace of the distinctive head which is characteristic of *Colpidium Colpoda.*

If the exposure is slightly greater, changes of a different kind are set up. The vacuole becomes greatly enlarged, with the result that the organism becomes pear shaped, or in extreme cases almost globular, the greater part of the globule being occupied by the distended vacuole. These changes develop gradually, and life may be prolonged for several hours after the completion of the exposure. If the exposure is not too severe, some individuals may survive the dose and resume gradually their normal shape. Under a high power magnification (1/12th inch) it was evident that these changes were accompanied by internal changes in the substance of the organism which it is not within my competence either to discuss or describe. In general, however, it may be stated that the irradiated protozoa appeared less transparent and distinctly more granular than the normal. It seems possible that an expert investigation of these changes might provide some interesting results for the cytologist.

A very considerable number of experiments, involving several hundred separate exposures, and extending over several months, were made to investigate what proportion of the exposed Colpidia would survive a given dose for one, two, or more hours. Some of these results are reproduced in the curves of fig. 2 (p. 400). It will be seen that they are of the same general shape as that of fig. 1. It will, however, be convenient to postpone a detailed consideration of these results to a later section of the paper.

Recovery of Colpidium Colpoda *from the Effects of Irradiation.*

In general the intensity of the radiation from the X-ray tube was maintained as nearly as possible at a constant value, namely, the maximum safe output of the tube. Experiments made with less intense radiation, and, therefore, requiring a longer time for the same dose, indicated that the time of exposure as well as the total dose had a marked effect on the result of the irradiation. These results could best be explained on the supposition that the Colpidia recovered rapidly from any dose which was less than lethal, and experiments were made to obtain some estimate of the effect.

It was found that, with a normal culture, a dose of 2 microcoulombs would produce a perceptible proportion of deaths in the course of two or three hours after exposure, and a dose of 3 microcoulombs would produce immediate death in a considerable proportion of the organisms. A series of observations was made in which the effect of doses of 1·5 microcoulombs, repeated after definite intervals of rest, was investigated. With doses of this magnitude, and with two-hour intervals between the doses, three exposures were required before any deaths occurred. The effect produced by the total dose of 4·5 m.c. spread out in this way over rather more than four hours was equivalent to a single dose of about 3 m.c. With an interval of three hours between the doses no effect was produced by the irradiation, even though four doses were given in a single day, and repeated on the same slide on the following day.

These experiments indicate that, if the Colpidia are exposed to a dose of radiation which is less than lethal, a partial recovery takes place within two hours, and practically complete recovery in three hours. To put the matter in a somewhat different way, if the rate of application of the rays is less than about 37,500 e in three hours, or about 200 e per minute, no lethal effects would be produced. This is a much higher intensity than has been hitherto employed in experiments of this kind.

Other Conditions affecting the Action of the Rays.

It must be understood that the numerical values quoted in the previous sections are mean values derived from a considerable number of individual experiments. Observations made on from 20 to 30 individuals in a single drop often differed considerably from the mean. Such variations are, in fact, demanded by the theory of the phenomenon proposed in a subsequent section. It is easy to show that, with the number of specimens usually present in a test drop, half the observations made on single drops should differ from the mean by more than 20 per cent., and occasional observations differing by as much as 50 per cent. from the mean are to be expected. In the preliminary experiments, however, the frequency of the occurrence of these unusually large or unusually small values exceeded the expectations, and a considerable amount of time was spent in attempting to identify and eliminate the possible disturbing factors.

The temperature of the laboratory during the experiments was found to be without effect on the results, at any rate over the normal range from 15° C. to 21° C. So also was the age of the culture between the limits of 5 and 18 days. Very old cultures, showing marked deterioration, however, were distinctly more easily killed by the radiation.

Experiments made with the tube excited at much below its normal voltage indicated that the wave-length of the radiation employed had a considerable effect on the results. With an alternative spark gap of 1·2 cm. or a peak potential of about 35,000 volts, immediate lethal effects were produced by an exposure of only 1 m.c. as measured by the ionisation chamber. It would appear that the softer radiation is very considerably more effective than the harder radiation. The experiments, however, are not conclusive. The Colpidia were exposed on a glass slide and were in a medium containing small but appreciable percentages of various inorganic salts. It is not certain that under these circumstances the indications of the ionisation chamber afford a reliable measure of the actual energy absorbed by the organisms, if the wave-length of the radiation is changed. The intensity of the radiation from the tube at these low voltages is much less than at the standard voltage, and the length of exposure required was inconveniently long. The effect of a change in wave-length can only be investigated satisfactorily with less highly resistant material. The results quoted in this paper all refer to a tube excited at the standard P.D. of 50,000 volts.

The susceptibility of the Colpidia to X-rays is certainly influenced by the length of time which elapses between the preparation of the slide and the time of exposure. A drop which has been on the slide for twenty-four hours before exposure will withstand a dose 20 per cent. greater than the normal, and a drop four days old a dose 50 per cent. greater than the normal, as judged by the immediate lethal effect. This is no doubt due to the fact that the Colpidia take some time to become thoroughly acclimatised to their changed conditions. Although the change of environment does not produce any visible effects on the Colpidia, it apparently produces some lowering of the vitality which renders the culture more susceptible to adverse influences. Under these circumstances it might have been advisable to prepare the slide two or three days before the actual exposure to the radiation. Considerations of time rendered this course impossible, and a standard interval of one hour was adopted between the preparation of the slide and its exposure to the rays.

Numerical Results.

The primary object of the experiments was to obtain numerical estimates of the proportion of Colpidia surviving measured doses of X-radiation. After the preliminary survey described above a standard procedure was adopted, which it may be advisable to recapitulate, as any variation of the conditions may affect the numerical values obtained, though not the general form of the relations. All the specimens used were descendants of a single individual of *Colpidium Colpoda.* The cultures were kept in a thermostat at a temperature of 19° C. until required for use. A single drop of the culture, from six to seven days old, was placed, by means of a sterilized glass pipette, on a glass counting chamber $\frac{1}{10}$ mm. deep, and was surrounded by a ring of small drops to prevent evaporation. A glass cover-slip 0·13 mm. thick was placed over the chamber and waxed into position. The drop normally contained from 20 to 30 individuals. It was left for one hour, and if it then appeared normal it was exposed at a distance of 2·50 cm. from the focal spot of an X-ray tube with a molybdenum anticathode, excited with a current of 7 m.a. at a P.D. of 50,000 volts. The radiation passed through an aluminium window 0·1 mm. thick and through the glass cover-slip. No other filtration was used. The radiation was measured by an air-gap ionisation chamber, and the exposures are recorded in the following tables in terms of the number of microcoulombs conveyed across this air gap during the exposure. A reading of 1 microcoulomb across the air gap is equivalent to 25,500 *e* units falling on the specimen.

Table I, giving the percentage surviving at the completion of a given dose, contains the combined results of the two sets of experiments plotted in fig. 1. In Table II each figure is the mean of six or more separate exposures, and includes observations made on about 200 individuals. The probable error in this table is, therefore, of the order of 7 per cent. The three sets of figures are plotted in fig. 2. Each graph is a sigmoid curve.

Table I.—Immediate Effect of X-rays on *Colpidium Colpoda*.

Exposure in m.c.	Percentage Survivors.	
	Observed.	Calculated.
1·08	100	100
1·90	100	100
2·17	100	100
2·31	98	99
2·72	87	89
3·12	64	63
3·26	50	51
3·42	32	37
3·96	10	9

Table II.—Delayed Action of X-rays on *Colpidium Colpoda*.

Exposure in m.c.	Percentage Surviving intervals of		Calculated for 1 Hour.
	1 Hour.	2 Hours.	
1·00	100	100	100
1·50	100	98	100
2·00	98	90	98
2·50	74	65	77
3·00	43	39	38
3·50	12	8	7

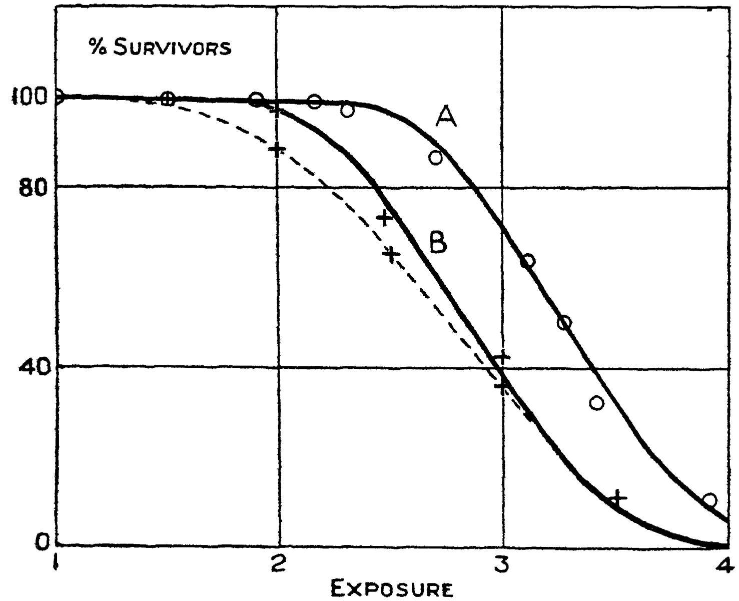

Fig. 2.

Discussion of Results.

The experimental curves are not of a type so simple that they can be identified by inspection. The form we attempt to assign to them will depend on the theory adopted as to their cause. It has been pointed out in the introductory section of the paper that certain important structures in the living cell are so small that the probability of their not being affected by the passage of a considerable dose of X-radiation falling upon the medium containing them is quite appreciable. If a single "hit" made by the rays on the given structure produces the death of the cell, the number of cells surviving will decrease exponentially as the dose increases. Let us suppose that instead of a single hit being sufficient to produce a lethal effect a succession of n hits on the same particle is required. Strictly speaking, we ought to include a time factor, for our experiments show that the organism has definite recuperative powers, so that if the interval between successive hits is sufficiently long no lethal effect will be produced. The recovery is appreciable, though far from complete in two hours. It is almost certainly negligible in the time taken to produce immediate lethal effects in the culture, and probably comparatively negligible in an interval of one hour.

Let N be the number of organisms initially present, and let λ be the probability that an effective hit will be made by unit dose of X-rays. The number of particles which have not been hit by a dose q of radiation is given by $y_0 = \mathrm{N}\varepsilon^{-\lambda q}$. Let y_n be the number of particles which have already been hit n times at some given moment. The number which will be hit again by an additional dose dq is $\lambda y_n dq$. These pass into the state $(n+1)$. With the same increment in the dose, a number of particles $\lambda y_{n-1}\,dq$ which have already been hit $(n-1)$ times are hit again and pass into state n. Thus

$$dy_n = \lambda y_{n-1}\,dq - \lambda y_n\,dq.$$

The solution of this equation with the given initial conditions is

$$y_n = \mathrm{N}\frac{\lambda^n q^n}{n\,!}\varepsilon^{-\lambda q}.$$

If n hits are required to produce a lethal effect, the number Y surviving a dose q of radiation is the sum of all the organisms in those states for which the number of hits is less than n; *i.e.*,

$$\begin{aligned}\mathrm{Y} &= y_0 + y_1 + \ldots + y_{n-1}\\ &= \mathrm{N}\varepsilon^{-\lambda q}\left(1 + \lambda q + \frac{\lambda^2 q^2}{2\,!} + \ldots + \frac{\lambda^{n-1}q^{n-1}}{\overline{n-1}\,!}\right)\\ &= \mathrm{N}\left(1 - \frac{\Gamma_x(n)}{\Gamma(n)}\right),\end{aligned}$$

where $x = \lambda q$, $\Gamma(n)$ is the complete gamma function, and $\Gamma_x(n)$ the incomplete gamma functon. The ratio $\Gamma_x(n)/\Gamma(n)$ has been tabulated by Prof. Karl Pearson.

The ratio Y/N represents the probability of an organism surviving a dose q of radiation. If we were working with an infinite number of organisms, this would also be the proportion of the organisms surviving. It is, however, of the essence of the theory that a hit is a matter of chance. Individual observations made on small numbers of specimens would be expected to differ from this mean. The probable error of experiments including 10,000 specimens would be 1 per cent. If the number of specimens is reduced to 200, the probable error is rather more than 7 per cent.

With the assistance of Prof. Pearson's Tables, the calculation of Y/N presents no difficulty, when λ and n are known. In the present case neither is known, and we must proceed by trial and error. Curves are plotted for various assumed values of λ and n, and fitted to the experimental results which have previously been plotted on squared paper. Since our assumption is that all the hits are made on the same structure, it is essential to the theory that the value of λ shall be the same for all the curves.

The full curve A in fig. 2 above is the calculated relation for the values $n = 49$, $\lambda = 15$. The circles clustering round this curve are the experimental values from Table I. It will be seen that the theoretical curve fits the experimental results with considerable accuracy. The full curve B is drawn for the values $n = 42$, $\lambda = 15$. The crosses are the experimental values of the percentage of survivors one hour after exposure to the different doses. The agreement here is also satisfactory, though not so good as for the previous curve. This may be due, in part, to the fact that owing to the smaller amount of material used the probable error of these experiments is greater than that for the results from Table I. There is also the possibility that the recovery of the protozoa from the effects of the irradiation may be producing a small but appreciable influence on the results. The broken curve gives the percentage surviving at the end of two hours after the exposure. It is clearly impossible to fit these results into this family of curves. This is, however, only to be expected, since direct experiment shows that the Colpidia make a very marked recovery in an interval of two hours.

The apparently large value of λ is, of course, due to the fact that, for convenience, we are working with a very large unit of radiation. Since 1 microcoulomb in the electroscope indicates a dose of 25,500 e on the slide, the value of λ may also be given as 15/25,500, or $5 \cdot 9 \times 10^{-4}$ per dose of 1 e.

This is of the same order of magnitude as the value of λ deduced for the tissue cells in the experiments of Strangeways and Oakley. The size of structure demanded by the theory is thus very much the same for both sets of experiments. Whereas, however, a single hit was sufficient to produce the observed effect in Strangeways' experiments, in the present experiment a succession of 49 hits is required to produce the immediate death of the organism, while a rather smaller number, 42, produces injuries sufficiently great to cause death within an hour of the termination of the exposure. Providing that we may assume the existence within the cell of structures of the necessary smallness, the theory suggested in this and earlier papers seems to afford an adequate explanation of the survival curves for Colpidia and the mitosis curve for the tissue cells.

The fact that the survival curves have the shape predicted by this theory does not rule out other theories of the effect which might, on investigation, lead to similar relations. If, for example, we assume that on an average a Colpidium requires a dose of 3·26 m.c. to produce immediate death, but that owing to biological variations among the specimens some individuals require more and some less than the average, then if these variations from the mean follow the usual law of errors, we should get a survival curve (the measure of precision being suitably adjusted) which would not be distinguishable from the experimental curves, within the limits of accuracy of these experiments. The difficulty is due to the large value of n in these experiments. If material could be found for which a small number of hits was sufficient to produce a measurable effect, the discrimination would probably be possible. Experiments were made on another protozoon, *Glaucoma scintillans*, but as the results were very similar to those obtained with *Colpidium Colpoda*, and as the new organism was very difficult to keep alive on the counting slide, the experiments were abandoned.

We have not yet defined what is to be understood by a "hit" on the structure. The absorption of a quantum of X-radiation by an atom results in the expulsion of a high-speed electron. This in turn produces ionization in surrounding molecules until its energy is spent. If we assume that the ionization of one of the molecules of the structure is sufficient to constitute a hit, the calculation of the size of the structure from the measured value of the coefficient λ is comparatively simple, and is given in detail in a previous communication.* For the value of λ deduced from the present experiments the diameter of the particle, assuming it to be approximately spherical, would be $3\cdot5 \times 10^{-5}$ cm.

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* Crowther, *loc. cit.*

On the other hand, it is possible that only the more violent processes accompanying the actual absorption of a quantum of X-radiation by the structure is adequate to constitute a hit. In this case, assuming that each high-speed electron produces about 1,000 pairs of ions in its course, the diameter of the structure might be as large as $3 \cdot 5 \times 10^{-4}$ cm. or 1/300th mm. Structures of the necessary order of magnitude are certainly to be found in most cells. It is not within the province of a physicist to attempt to identify them.

It may be pointed out, in conclusion, that if the action of X-rays on the cell is due to their effect on the minuter structures in it, rather than on the mass of protoplasm as a whole, the fact of the quantum absorption of the rays necessitates a relationship between survival and dose of the kind derived in this section of the paper, and the fact that the experimental relation is consistent with that deduced supports this hypothesis. If the hypothesis is accepted, the determination of the size of the structure concerned from the coefficient λ of the effect may possibly lead to the identification of the structure, and may thus throw some light on the actions taking place in the cell. This problem, however, must be left to the cytologist.

The X-ray apparatus used in these experiments was purchased out of a grant made to me by the Government Grants Committee of the Royal Society, to whom I express my thanks. I also acknowledge my deep indebtedness to Mr. W. Buddin, who has kindly prepared all the cultures used in the experiments, and to Mr. Ward Cutler and to Dr. Peters for material supplied.

Reports from the Royal Society's Kala Azar Commission in China.

No. 6.—*Notes on the Species of Sandflies* (Genus Phlebotomus) *of North China.*

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(Communicated by Dr. H. H. Dale, Sec. R.S.—Received May 26, 1926.)

As in the case of most of the blood-sucking insects of North China, very little is known regarding the species of sandflies, and up to the present only one has been recorded. It was collected by Bolt at Wo-Fu-Ssu Temple, Western Hills, Peking, and at Tung-Chou, twelve miles east of Peking, and described by Newstead (1916) as a variety *chinensis* of *Phlebotomus major*, Annandale. In view of the recent observations of Young and Hertig (1926) on the development of the parasite of Chinese Kala Azar in this species, and in a second one which they refer to as species " C," it is of some importance to record our observations on the species of *Phlebotomus* known to us from North China.

As we arrived at the end of the sandfly season in 1925, we were only successful in obtaining a few specimens of *P. major* var. *chinensis* and some examples of a new variety of *P. perturbans* de Meijere. Recently, when on a visit to Peking, one of us (W. S. P.) had the opportunity, through the kindness of Mr. Hertig, entomologist to the Rockefeller Kala Azar Commission in China, of examining the three species collected by the members of that Commission. One of these had already been determined by Mr. Hertig as *P. major* var. *chinensis* ; their species " C " proved to be the variety of *P. sergenti* Parrot, described by Newstead, and species " B " is the new variety of *P. perturbans* de Meijere noted above.

Mr. Hertig kindly gave us many preserved specimens of the three species, which we have studied and compared with our own material. The following notes on these three species are recorded, in the hope that they may stimulate others to collect sandflies in China. We feel sure that when these small blood-sucking flies are better known, many more species will be found in this country.

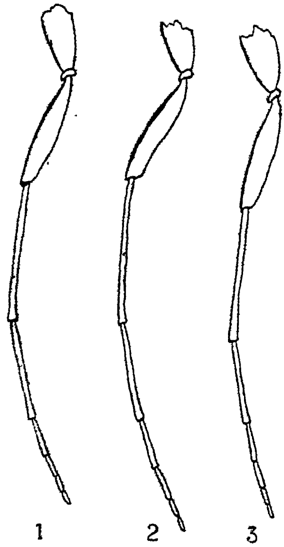
P. major var. *chinensis* Newstead.

A large yellowish species, measuring from 3 to 3·5 mm. in length, with dark hairs on wings, thorax and abdomen, those on the dorsal surface of the latter being erect. Palpal formula of both sexes, 1, 4, 2, 3, 5; segment 5 is about two and a half times the length of segment 4. Antennal formula of both sexes 2/III-XV.

The legs are very long in both sexes, particularly the hind pair. The first tarsal segment is about three times the length of the second (fig. 1).

The wing of the female (fig. 4) is relatively broad, the costal margin almost straight, with a gentle backward curve ending at the apex. The posterior margin is deeply arched up to the termination of vein Cu_2, and the wing is broadest at a line joining this point with one on the costal margin opposite it; from the end of vein Cu_2 the margin bends up to the base.

Vein R_1 overlaps the origin of vein R_2 by about a quarter of the length of the latter. Vein R_2 (anterior branch of the second long vein) is almost double the length of the portion of the radial stem between the origins of veins R_2 and R_3, and that of vein R_4 (the distance between the forks of the second long vein). The origin of vein R_4 is a little nearer the base of the wing than that of the branches of the media.



FIGS. 1, 2, AND 3.

The posterior border of the wing of the male is a little less arched than that of the female. Vein R_1 overlaps the origin of vein R_2 by about one-third the length of the latter. Vein R_2 is almost three times the length of the portion of the radial stem between the origins of veins R_2 and R_3, and that of vein R_4. The origin of vein R_4 is almost exactly opposite that of the branches of the media.

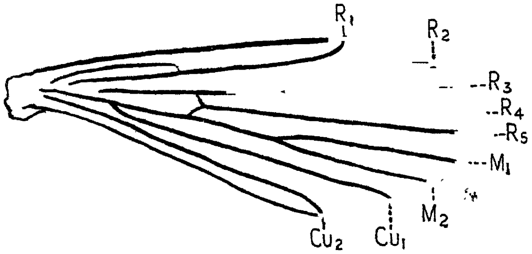


FIG. 4.

The male terminalia (external genital apparatus, hypopygium) are similar to those of *P. major*. The claspers are long and rather narrow. The distal segment of the superior clasper is relatively short, about two-thirds the length of the proximal segment, which is long and narrow. It is armed with five long macrochætæ, which are arranged as follows :—Two are apical, two are situated close together about the middle of the inner border, and the fifth is attached to the external border, a little nearer the middle than the base of the segment. Newstead notes that the basal segment is relatively larger than that of *P. major*.

The inferior claspers are long and slender, but are not armed with macrochætæ. The intermediate appendage is not lobed.

This species is widely distributed in North China. We have examined specimens from several localities in Shantung, and from Tientsin and Peking in Chihli. Young and Hertig record it from Hsüchowfu in Kiangsu. In Peking it is a pest well known to residents, and is said to be very common at the end of May and June. It is worthy of note that Kala Azar is a comparatively rare disease in the capital.

In view of the fact that Young and Hertig have found the parasite of Chinese Kala Azar develops in a large percentage (85·3) of females of this species, fed on the blood of giant hamsters (*Cricetulus triton*) infected with the parasite, and the general belief that Kala Azar is either very rare, or not endemic, south of the Yangtse River, it is of some importance to ascertain the southern limit of the distribution of *P. major chinensis*.

As we have not yet been in China during the sandfly season, we have not had an opportunity of studying the seasonal prevalence, number of broods, distribution, etc., of this species. Young and Hertig, as the result of their observations on this species during the sandfly season of 1925 at Hsüchowfu, state that : " It seems to be single-brooded, being found chiefly during June and the first part of July. Only a small fraction of these sandflies completed their cycle during the summer and, as a result, laboratory bred material of this species has been scanty." We certainly found it very rare in Tsinan and Taian at the end of July, and during August and September.

Of the few females collected in August and up to September 12, only two laid eggs. One batch of these proved to be sterile, and from the other two larvæ hatched after an interval of from 9 to 10 days. Both larvæ died on November 28. Although prolonged attempts were made to feed the caught females under the most favourable conditions, they all refused to bite.

P. sergenti, Parrot var. Newstead.

A medium-sized, pale yellow species, measuring from 2 to 2·5 mm. in length, with lighter hairs than those of *P. major chinensis*; those on the dorsal surface of the abdomen are erect. Palpal formula of both sexes, 1, 4, 2, 3, 5. Antennal formula of male and female, 2/III–XV.

The legs are long and the second tarsal segment is about half the length of the first (fig. 2). The wing of the female (fig. 5) is distinctly narrower and more lancet-shaped than that of *chinensis*, and the posterior border is less arched. These characters are of considerable use in separating the female of this species from that of *chinensis* in the living condition.

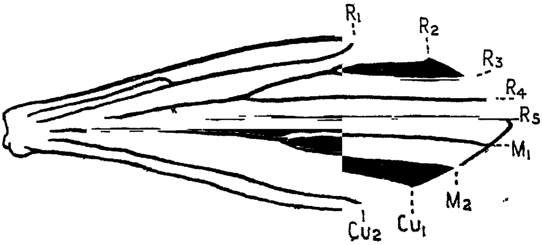


FIG. 5.

Vein R_1 overlaps the origin of vein R_2 by about one-third the length of the latter. Vein R_2 is equal to the length of the portion of the radial stem between the origins of veins R_2 and R_3, and that of vein R_4. The origin of vein R_4 from the radial stem is much nearer the base of the wing than that of the branches of the media. Vein M_1 is longer than the corresponding vein of the wing of *chinensis*. It should be noted that these characters can only be satisfactorily made out in mounted wings owing to the thick covering of hairs in living specimens.

The wing of the male is narrower than that of the female. Vein R_1 overlaps vein R_2 by about a quarter of the length of the latter. Vein R_2 is slightly longer than the portion of the radial stem between the origins of veins R_2 and R_3, and that of vein R_4.

The male terminalia are large and prominent, but less so than those of the male of *chinensis*. The segments of the superior clasper are shorter and stouter, especially the proximal segment, and the distal segment is armed with one slender and three stouter macrochætæ. The most striking difference between the terminalia of the two species is the presence of a brush of short, non-deciduous hairs on the internal surface of the proximal segment of the superior clasper of the male of *sergenti*.

P. sergenti is a European species, and is mainly found along the Mediterranean littoral. It has been recorded from localities in the south of Spain and Portugal, from Algeria, the Caucasus at Tiflis, from North Persia, and from the North-West Frontier Province in India. Newstead records this variety of *sergenti* from Amara, Mesopotamia. He notes that it differs from the typical *P. sergenti* Parrot in having the third segment of the antenna shorter, and the bifurcation bearing the two macrochætæ at the end of the distal segment of the superior clasper markedly unequal in length.

In China, Young and Hertig record *P. sergenti* var. (species " C ") from Peking and Hsüchowfu, and at the latter place they note that it was common from the middle of June to the end of September. As a result of their breeding experiments they conclude that this species has two or more broods in the summer. We were not successful in capturing any specimens of *sergenti* in Shantung in 1925.

As already noted, Young and Hertig found flagellates in about 3 per cent. of females of this species fed on infected giant, and striped, hamsters.

P. perturbans de Meijere, var. nov.

Male.—A medium-sized dark brown species with dark recumbent hairs on the dorsal surface of the abdomen, and measuring from 2 to 2·2 mm. in length. Palpal formula 1, 2 (3, 4), 5 ; segment 5 is long and slender, and is about equal in length to segments 3 and 4. Antennal formula 1/III–XV.

The legs are long. The second tarsal segment (fig. 3) is about half the length of the first. The wing (fig. 6) is lancet-shaped, and the posterior border is not more arched than the anterior. Vein R_1 overlaps vein R_2 by about half the length of the latter. Vein R_2 is equal in length to the portion of the radial stem between the origins of veins R_2 and R_3, and that of vein R_4. The origin of vein R_4 is much nearer the base of the wing than the origin of the branches of the media.

The male terminalia are not particularly prominent. The proximal segment

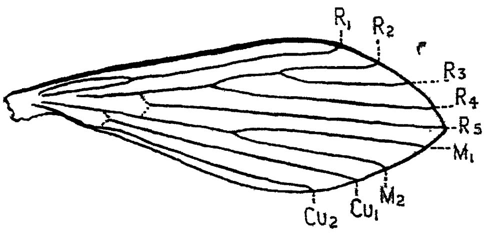


FIG. 6.

of the superior clasper is long and very broad. The distal segment is half as long as the proximal segment ; it is narrower and has a marked tubercle about two-thirds the distance from the base. It is armed with four long macrochætæ, two apical and two situated close together on the tubercle ; one of the apical macrochætæ is spathuliform (fig. 7).

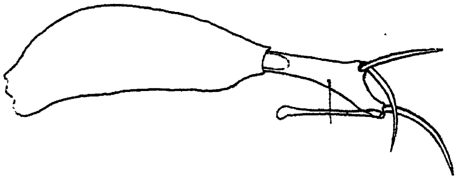


FIG. 7.

The intermediate appendage is about two-thirds the length of the basal segment of the superior clasper, and is bluntly rounded at the tip. The intromittent organ consists of the usual two dark, pointed, spine-like structures ; the genital filaments are protruded. The inferior clasper is as long as the basal segment of the superior clasper, and is slender. The lamellæ are of the usual type.

Female.—The female is similar in colour to the male, but if anything it is darker, the hairs markedly so, the recumbent hairs on the abdomen forming a thick layer. Palpal formula 1, 2, 3, 4, 5, and segment 5 is longer than segments 3 and 4 together. Antennal formula 2/III–XV. Legs as in the male.

The wing (fig. 6) is broader than that of the male. Vein R_1 overlaps vein R_2 by about two-thirds the length of the latter. Vein R_2 is longer than the portion of the radial stem between the origins of the veins R_2 and R_3, and that of vein R_4, and the latter portion is equal to the part of the radial stem between the origin of vein R_4 and vein R_5. The origin of vein R_4 is nearer the base of the wing than the origin of the branches of the media.

The male terminalia of this species are very similar to those of the Oriental *P. perturbans* de Meijere as described by Annandale (1910), and later by Sinton (1924). Owing to de Meijere's (1909) meagre description of his Javanese species *P. perturbans,* it is not possible to compare the Chinese species with it. Sinton points out that there is some doubt as to whether *P. perturbans*, as described by Annandale, is identical with the Javanese species, and that before this can be settled it will be necessary to examine de Meijere's type, which is in Amsterdam. As the Chinese species differs in some of its characters from the Indian *perturbans,* it will be best in the meantime to consider it a new variety.

This Chinese variety of *P. perturbans* can easily be distinguished from the other two species by its smaller size, darker colour, recumbent hairs on the abdomen, the characters of the venation, and those of the male terminalia.

This species was found in Tsinan and at Taian, being common in the latter place, and especially in a temple on Taishan, during July and August; as far as could be ascertained it was biting man. About 30 females were collected from Taian in August, and these laid a number of eggs from which larvæ hatched in due course. Two of these pupated on September 22 and 25 respectively, and one female fly emerged on October 5. As all attempts to get the caught flies to feed on man failed, the female which had hatched in the laboratory was placed in a glass chimney, into which a gecko was suspended in a small wire cage, but it refused to feed on the gecko.

Many more females were caught in Taian during the latter part of August, and from these about 50 eggs were collected. The larvæ started hatching from September 5, and from one of these which pupated later a female fly emerged on October 15. This female also refused to feed, either on a gecko or on man. The remaining larvæ in this and in the previous experiment died towards the end of November.

During the course of these rearing experiments it was noted that the larva of this species could withstand a considerable amount of cold and desiccation and yet remain quite active. It was also noted that once a larva pupated, the adult invariably hatched out about 10 days later.

A large number of larvæ were accidentally destroyed by some small predacious beetle larvæ which gained entrance to the breeding receptacles. The breeding technique employed was a modification of that suggested by Smith (1925). The larvæ seemed to thrive on rabbit's as well as on gecko's fæces, which were kept moist by standing the receptacle in a dish of water. Although we have not been successful in keeping the larvæ of this species alive during the winter, we have little doubt that *Phlebotomus* hibernates in North China in the larval stage.

Young and Hertig note that *P. perturbans* var. is a rare species in Hsüchowfu, and though they state it is known to feed on man in nature, they failed to get 70 females to feed, either on man or on the hamster.

The drawings illustrating these notes were executed by Mr. Feng Lang Chou.

NOTE ON THE TRANSPORTATION OF P. ARGENTIPES FROM INDIA TO CHINA.

It is generally assumed that considerable difficulties attend the breeding and transportation of sandflies, but the results of an attempt made by one of us (E. H.) to transport *P. argentipes* Annandale and Brunetti, from Calcutta to

China, suggest that these difficulties may have been exaggerated. Through the kindness of Dr. L. E. Napier and Military Assistant Surgeon R. O. A. Smith, larvæ and also eggs of *P. argentipes* were obtained at the Calcutta School of Tropical Medicine on July 30, 1925. These were kept on board ship, and supplied with food and moisture under the very unfavourable conditions of an ordinary ship's cabin during the hot weather. The larvæ pupated and adult sandflies emerged 11 to 13 days later. At Singapore, and also on board ship between there and Hongkong, the adult sandflies were fed, and the females laid eggs on moist plaster of Paris provided for them. At Hongkong, owing to an accident, the eggs could not be attended to for three or four days, and, as a result, they were not kept sufficiently moist. Moreover, on the journey between Shanghai and Tsinan, travelling conditions prevented any attention being given to the young larvæ, which were just beginning to emerge from the eggs. In spite of these very unfavourable conditions, about eleven larvæ hatched out, and two of these completed their development in the laboratory at Tsinan, the adults emerging on October 10. Unfortunately, they were both females, and in the absence of males refused to feed, so we were not able to rear another brood from them.

In view of the very unfavourable conditions under which the eggs and larvæ were kept during the latter part of the journey, and also the extreme temperatures to which they were exposed on one occasion (108° F.), it is rather surprising that any of the insects completed their development. It is very evident that in the case of *P. argentipes*, if one started with a larger number of eggs and larvæ, there would be no great difficulty in transporting it from one country to another.

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The Effect of Radium on Mitosis in vitro.

By R. G. Canti, M.D. (Cantab.), and M. Donaldson, F.R.C.S. (Eng.).

(Communicated by Sir Frederick Andrewes, F.R.S.—Received October 6, 1926.)

(From the Laboratories of the Cambridge Research Hospital.)

The investigations recorded in this communication were carried out as part of an enquiry into the causes of the disappearance of certain types of new growth under the influence of gamma radiation.

One of us (R. G. C.) had already investigated the histological changes produced by radium in carcinoma of the cervix uteri,* but it was felt that further progress was more likely to be made along lines where the methods of investigation were less limited. To this end it was decided that tissue cultures *in vitro* would afford material on which more direct observations of the changes in the individual cell could be made. The cultures—supplied to us by our colleague, T. S. P. Strangeways—were obtained in some cases from the sclerotic and choroid and in others from cartilage of embryo chicks of 6 to 8 days' incubation. These cultures were of the 2nd passage, incubated at 38° C. for 18 to 24 hours after implantation. They were cultivated in fowl plasma and chick embryo extract, upon a No. 1 coverslip of soda glass over a hollow-ground glass slide. They were of uniform growth, a considerable number of mitotic cells being present at the time of observation. In the early experiments the stock cultures were used as controls, and in the later experiments a certain number of the experimental cultures were set aside for this purpose.

Cells in mitosis usually varied between 10 and 40 at any one time in each culture.

The results recorded in the present paper are confined to observations on the effects of irradiation upon mitosis. Cytological changes other than those recorded were noted, for example, the breaking up of the cells, and abnormal forms of mitosis which corresponded closely to the results obtained by Strangeways and Oakley† when working with X-rays.

The radium at our disposal consisted of two tubes of platinum, with walls 0·5 mm. thick, each containing 50 mgm. of radium element. In the experiments in which observation of the cells during the actual period of irradiation was not desired, the cultures and radium were placed in a wooden rack inside a lead box in a biological incubator, the distance between the radium and the culture varying in the different experiments. The normal stock cultures and controls were incubated in a room some distance from the laboratory in which the radium experiments were being carried out.

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* Donaldson, M., and Canti, R. G., 'B. M. J.,' vol. 2 (July 7, 1923).

† Strangeways, T. S. P., and Oakley, H. E. H., 'Roy. Soc. Proc.,' B, vol. 95 (1923).

Observations on these cultures before and after irradiation and on the controls were made in special wooden incubators heated with electric bulbs controlled by capsule thermostats. These incubators contained microscopes which could be manipulated through curtained doors on either side. The light used for observation was a 60-watt. Fullolite lamp. The lenses were an Abbe condenser with iris diaphragm, a two-thirds or one-sixth inch objective and No. 4 Huyghenian or No. 10 " telaugic " eyepiece.

Other experiments were conducted in which direct microscopic examination was carried out during the period of irradiation. In these experiments the radium was placed in a special superstage attachment (fig. 1). This stage consists essentially of a plate of metal ¼ inch thick, having a central aperture about 1 inch in diameter. The plate is attached to the moving stage of the microscope so that the aperture permits the light to pass from the condenser to the objective. The tissue-culture preparation mounted on a hollow-ground glass slide was placed on the top of the superstage, and held in position with the usual clips. The two radium tubes were placed horizontally, each at 1·4 cm. from the culture, in slots in the superstage on either side of the aperture, this being the nearest distance that the excursions of the substage condenser would allow.

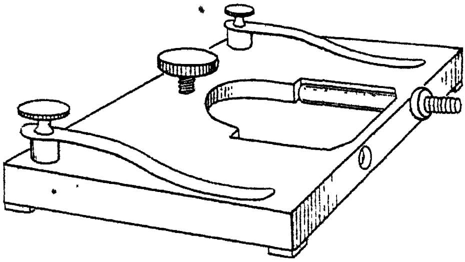

Fig. 1.—Radium superstage.

For the protection of the workers the following precautions were taken. The incubator was made of lead 2 inches thick below and on the side nearest the observer, and 1 inch thick elsewhere. The focussing adjustments and mechanical state were manipulated entirely from the outside. A special attachment made to our design by Messrs. Swift was added to the draw-tube of the microscope so that the rays of light from the objective were reflected from a polished steel mirror and viewed at an angle. This necessitated increasing the tube length of the microscope, compensation for which was made by adding an Ainslie's correcting lens to the back of the objective. The steel mirror was attached to a swivel, so that when swung out of position the beam of light could pass vertically upwards to a photographic attachment, if so desired (fig. 2).

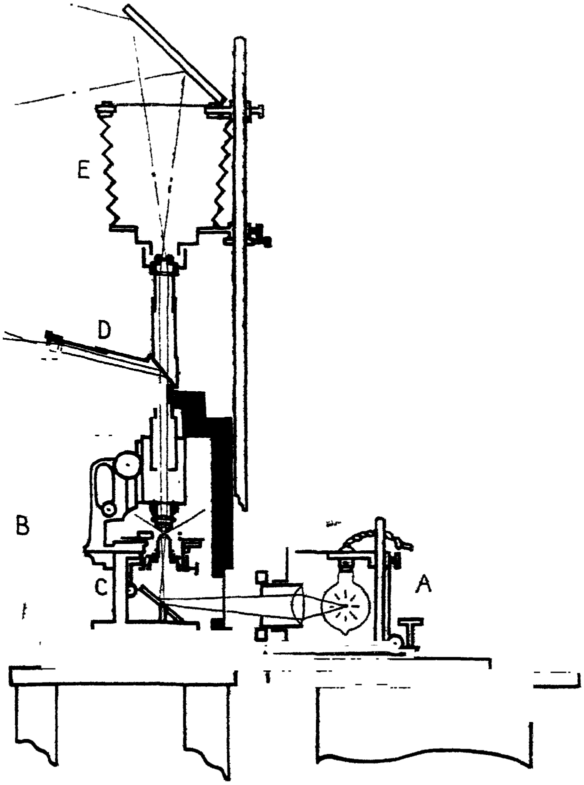

Fig. 2.—Section of apparatus set up for use. A, lantern; B, incubator; C, microscope; D, side-view attachment; E, camera.

In certain observations it was decided to record the number of cells undergoing mitosis each quarter of an hour. In these observations only those cells in telophase were counted. This stage was selected because it is easily recognisable, and owing to its normally short duration it was unlikely that the same cell would be counted on more than one occasion.

Experiment I.—In this set of observations 100 mg. of radium element filtered with 0·5 mm. of platinum were placed directly on the coverslip in as close proximity to the cultures as possible, and left in position for various times. The cultures were then fixed and stained immediately. Four slides were kept as controls and showed mitosis throughout the whole of the 12 hours (Table I).

Table I.

Culture.	Hours.	Presence or absence of mitosis immediately after irradiation has ceased.
R 16	1	Present.
R 18	2	Absent.
R 20	2½	,,
R 22	3	,,
R 7	3	,,
R 8	3	,,
R 11	12	,,

Result.—Under these conditions it will be seen that mitosis is present after an hour, but is absent after two hours, and subsequently up to 24 hours.

Experiment II.—In this set of observations 50 mg. of radium element filtered with 0·5 mm. of platinum were placed at a distance of 1·4 cm. from the cultures and left in position for various times. The cultures were then fixed and stained immediately. Four slides were kept as controls and showed plentiful mitosis (Table II).

Table II.

Culture.	Hours.	Presence or absence of mitosis immediately after irradiation has ceased.
HA	3	Present.
HB	3	,,
HC	3	,,
HD	3	,,
HE	4	,,
HG	4	,,
HF	7	Absent.
HH	7	,,

Result.—Under these conditions, using less radium at a greater distance than in Experiment I, mitosis is present after 4 hours, but absent after 7 hours.

Experiment III.—In this set of observations 100 mg. of radium element filtered with 0·5 mm. of platinum were placed at a distance of 1·4 cm. from the culture and left in position for 3 hours. The number of mitoses (in telophase only) were counted on two occasions before the radium was placed in position, and from time to time during 4 hours after the radium was removed (Table III).

Table III.

Culture F.B.O.

Times of Observation.	No. of Cells in Telophase.
1.45 to 1.55	7
2.45 to 2.55	10
Radium in position from 3.30 to 6.30	
7.10 to 7.25	0
7.30 to 7.40	0
7.40 to 7.50	0
7.50 to 8.0	1
8.0 to 8.10	0
8.10 to 8.20	1
8.45 to 9.0	2
9.0 to 9.15	2
9.30 to 9.40	4
10.30 to 10.45	5
10.55 to 11.0	5

At 11.5 the culture was fixed and stained and found to contain nine cells in telophase.

Result.—Under the conditions of this experiment it is seen that mitosis is found to be absent after irradiation for 3 hours, but gradually returns in the course of a few hours, after the removal of the radium.

In *Experiment IV* the cultures were placed in position on the superstage of the microscope in the lead incubator, and counts were made of the number of cells in telophase during each successive quarter of an hour. After 2¼ hours the two tubes containing together 100 mg. of radium element filtered with 0·5 mm. platinum were placed in the slots in the superstage each at a distance of 1·4 cm. from the culture. Counts were continued as before. The radium was left in position for 6 hours and the observations continued for another 3½ hours.

An unirradiated culture was used as a control. In it the total number of cells which appeared in telophase during a quarter of an hour were recorded as before. Ten such counts were made during a total period of 11 hours and these have been superimposed on the accompanying chart of the irradiation culture.

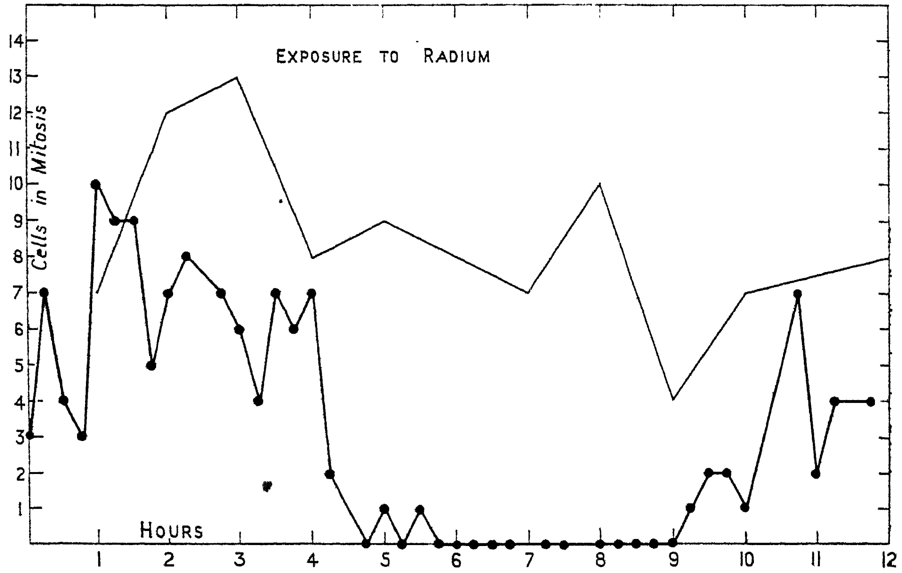

Chart of Experiment IV, showing number of cells in telophase. Thin line superposed = cells in telophase in unirradiated control.

Result.—In this experiment it is seen that, under the particular conditions employed, mitosis continues for two hours after the commencement of irradiation, then ceases almost abruptly and remains absent till approximately two hours after the removal of the radium, when it returns. The unirradiated control culture serves to show the gradual falling-off of the number of cells in mitosis, which may be expected in any culture as it grows old.

Conclusions.

The first experiment shows that it was possible to bring about the cessation of mitosis in tissue cultures *in vitro* by means of radium irradiation.

The second experiment shows that with a smaller intensity a greater time was necessary to bring about this effect.

The third experiment shows that, with the intensity of radium irradiation employed, not only did mitosis cease, but that, when the radium was removed after an exposure of several hours, mitosis reappeared.

The fourth experiment confirmed this fact, and further showed that at the commencement of irradiation not only did cells complete division when mitosis had already commenced, but also that some cells actually began mitosis and went through the process of division in an apparently normal fashion.

Another observation brought out by the graph is a sudden drop in the number of cells in mitosis. This drop occurs two hours after the commencement of irradiation with the particular intensity employed.

In none of these experiments was there any evidence of the radium producing an increase in the number of cells in mitosis.

The expenses in connection with this study were met by a grant from the Medical Research Council.

The Chemical Nature of Hæmochromogen and its Carbon Monoxide Compound.

By R. Hill, M.A.

(Communicated by Sir Frederick Hopkins, F.R.S.—Received May 31, 1926.)

(From the Biochemical Department, Cambridge University.)

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I. *Introduction.*

In a previous communication (Hill, R., 1925) I summarised the properties of a number of derivatives of the blood pigment in which the iron was replaced by different metals. When iron combines with hæmatoporphyrin to give a hæmatin, the spectrum resembles that of a typical metallo-porphyrin only when the pigment is in the form of hæmochromogen.

The nature of hæmochromogen has until quite recently not been understood. The work of Anson and Mirsky (1925) shows that hæmochromogen, prepared directly from oxyhæmoglobin by reduction in alkaline solution, is a compound between the pigment and the protein. Again, these workers state clearly that when other simpler substances are used instead of the protein, each substance gives its characteristic hæmochromogen by actual combination. They emphasised the fact that all substances capable of yielding typical hæmochromogens contained nitrogen, and showed that hæmochromogen is dissociated in solution into pigment and free nitrogen compound, to a greater or less extent depending on the particular hæmochromogen in question. Dilling (1910) had made a detailed qualitative study of hæmochromogens prepared by means of different reagents, but he does not seem clear as to the part played by the reduced hæmatin in the formation of hæmochromogen. The nearest approach to a solution of the problem previous to Anson and Mirsky's work was that of Bertin Sans and de Moitessier (1893). These authors had shown that pure hæmatin when reduced with sodium hydrosulphite will not give hæmochromogen, unless albumen or certain amines were added. They failed, however, to exclude the possibility of the relation between pigment and nitrogenous substance being other than chemical combination. Von Zeynek (1910) analysed pyridine hæmochromogen and found that it contained 2·2 molecules of pyridine. He regarded it as a compound of hæmochromogen and pyridine. This work, therefore, gave no indication of the minimum amount of pyridine necessary to convert reduced hæmatin into hæmochromogen.

The present work was undertaken in order to see if any simple chemical relationship existed between pigment and nitrogen compound in the formation of a hæmochromogen, as defined by its spectrum. Reduced hæmatin in the absence of suitable nitrogen compounds has a diffuse spectrum and a dull purple colour. Hæmochromogen, on the other hand, has a sharp two-banded spectrum and is bright red. Most of the other derivatives of hæmatoporphyrin have a hæmochromogen-like spectrum, and it was necessary to show that with metals other than iron nitrogeneous impurities do not influence the type of spectrum exhibited by the pigment.

II. *Metallo-Hæmatoporphyrins in Relation to Hæmochromogen.*

Starting with pure hæmin, hæmatoporphyrin was prepared by the method of Nencki, and recrystallised in the form of the hydrochloride from hydrochloric acid. The pigment was dissolved in glacial acetic acid and heated separately with the acetates of the metal, copper, nickel, and ferrous iron, together with sodium acetate. The copper and nickel produced red solutions showing two-banded spectra similar to hæmochromogen, the iron produced a brown solution with an acid hæmatin spectrum. On pouring the solutions into water the pigments were precipitated and could be washed free from acetic acid and metal acetates. The washed pigments were re-dissolved in dilute sodium carbonate, and the iron compound reduced with hydrosulphite. While the nickel and copper compounds still showed their typical two-banded spectra, the iron compound showed an ill-defined region of absorption similar to reduced hæmatin. On adding denatured globin, pyridine or ammonia, the iron compound gave hæmochromogen. Thus artificial iron hæmatoporphyrin shows the same behaviour as hæmin prepared directly from blood. Nickel and copper hæmatoporphyrin showed no observable change on the addition of denatured globin, and only slight shifts of the bands where an *excess* of pyridine or ammonia was added—a behaviour shown by free hæmatoporphyrin itself under the same conditions.

The iron compound of hæmatoporphyrin thus behaves exactly as hæmatin, and on reduction does not give the hæmochromogen spectrum shown by other metallic derivatives of hæmatoporphyrin, unless some nitrogenous substance is added. This fact made it appear that the hæmochromogen was simply the ferrous compound of the porphyrin corresponding with the divalent copper and nickel compounds. The nitrogen compounds were possibly only necessary for a complete reduction of the ferric iron of hæmatin, and played no part in the final hæmochromogen.

Iron hæmatoporphyrin, however, gives a different spectrum according to the reagents present when the pigment is reduced in alkaline solution, and this property is not shared by any other metallic hæmatoporphyrin so far examined. This is an indication that the iron atom alone confers on the pigment the property of forming molecular compounds with a large variety of substances when the pigment is in the reduced state.

III. *Reactions of Reduced Hæmatin.*

1. *Method of Experiment.*—In dealing with the reactions of the highly complex pigment hæmatin, one which, when reduced, reacts rapidly with oxygen, it is difficult to apply the ordinary methods of analysis. This paper deals with the reactions of the reduced pigment with pyridine, as the nitrogen-containing substance, and with carbon monoxide. For the estimation of the latter the methods used for hæmoglobin can be applied. Ferricyanide reacts at once, oxidising the pigment and liberating the carbon monoxide. This reaction was carried out in a Barcroft micro-respirometer, and I should like to take this opportunity of thanking Dr. D. C. Harrison for lending me his apparatus and giving valuable assistance in the actual experiments. In the case of pyridine I used

the simple method of adding this reagent, in known amount, to the pigment and observing the course of the reaction as indicated by the colour and by means of the spectroscope. By taking a range of different concentrations it was found possible to eliminate the dissociation factor of the pyridine compound, as above a certain concentration this becomes negligible. With other nitrogen compounds, such as ammonia and piperidine, which are more easily dissociated from the pigment, the method is not applicable because the necessary high concentrations of the pigment cannot be reached.

The simple experiment described below is based on the observations of Bertin Sans and de Moitessier (1893). They describe the change in spectrum produced in pure carbon monoxide-reduced-hæmatin when ammonia or albumen is added. It is suggested that carbon monoxide-hæmochromogen, produced in the presence of a nitrogen compound, more nearly resembles carbon monoxide-hæmoglobin than the carbon monoxide compound in the absence of any nitrogen compound; but this is contrary to the actual statement of the above workers on the pigment.

The characteristic properties of the three compounds under investigation, namely, carbon monoxide-reduced hæmatin, CO-pyridine hæmochromogen and pyridine hæmochromogen, will be made clear in what follows.

2. *Characterisation by Means of Absorption Spectrum.*—Hæmin dissolved in dilute sodium hydroxide was reduced with sodium hydrosulphite. The spectrum of the purple-brown solution showed two faint bands. The solution was then saturated with coal gas. A change in colour to pure carmine red immediately took place, and the spectrum resembled hæmochromogen in showing two distinct bands, the α-band being much the darker. This shows that even with pure hæmatin a hæmochromogen-like spectrum can be produced apart from any nitrogen-containing substance. On adding a very dilute (0·25 per cent.) solution of pyridine, the two bands moved towards the red end of the spectrum and became more equal in intensity. Finally they became stationary and the spectrum closely resembled that of CO-hæmoglobin. One drop of pure pyridine was added and immediately the spectrum changed to that of ordinary pyridine hæmochromogen, the two bands then being farther towards the violet end of the spectrum than in the solution without pyridine (see fig. 1). These changes in the position and character of the bands are fortunate, because by their nature they exclude any possibility of a mixed spectrum, and show the presence of at least three derivatives of reduced hæmatin. Now if pyridine hæmochromogen is shaken with carbon monoxide, the carbon-monoxide compound containing pyridine identical with the above is obtained; but if excess of pyridine

is present, the hæmochromogen will not react with carbon monoxide as long as the solution is alkaline.

We could explain this behaviour most simply by assuming that the carbon monoxide in CO-hæmochromogen is actually replaced by pyridine when hæmochromogen is produced. As will be seen below, this simple assumption is justified.

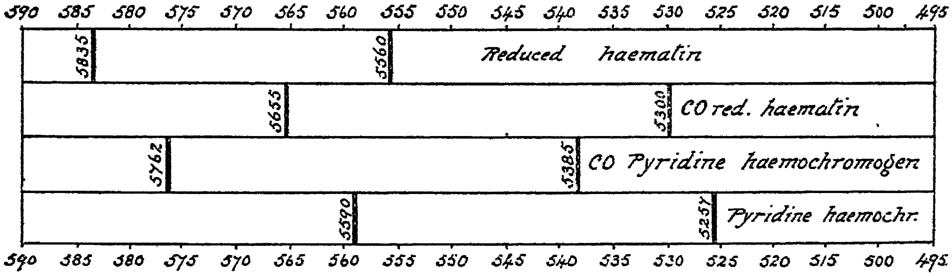


Fig. 1.

3. *Addition of Pyridine to Reduced Hæmatin in Strong Solutions.*—Pure pyridine was dried with solid caustic soda, distilled (114–115°), and a solution was made containing 2·50 gm. in a litre of water. The solution of hæmatin contained 1·03 gm. hæmin together with 1 gm. Na_2CO_3 in 50 c.c. water. Both solutions were then 0·0316 normal in terms of iron in hæmatin and nitrogen in pyridine. A reducing solution was prepared containing 20 gm. sodium hydrosulphite and 10 gm. sodium carbonate in 200 c.c. of water. The experiments consisted in adding to 1 c.c. of the hæmin solution definite volumes of pyridine solution and making up to a definite volume with water. After reducing with a given volume of reducer, the presence of uncombined reduced hæmatin was detected by: (1) the colour, by comparison with a similar solution containing an excess of pyridine; (2) by the spectroscope; and (3) in the case of very strong solutions by the appearance of the solid, which separates after reduction.

Hæmochromogen containing reduced hæmatin as a precipitate was found to have the characteristic blue-violet reflex of the latter, while hæmochromogen as a precipitate under the same conditions has a brown-red colour. The colour of the solution by daylight was found to be the most sensitive indication of the complete formation of hæmochromogen. In the table below is shown an experiment typical of the series, which is shown as a graph in fig. 2.

Total volume, 3·5 c.c. Reducer added, 0·3 c.c. Hæmatin, 1 c.c.

Pyridine.	Spectrum.	Colour.
c.c.		
1·8	+	+
1·9	−	+
2·0	−	−
2·1	−	−
2·2		

The presence of uncombined reduced hæmatin is shown by a plus sign.

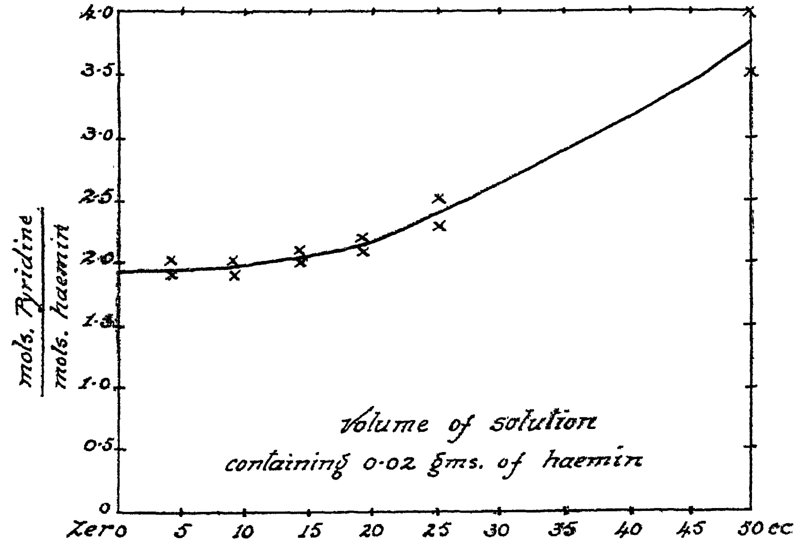


Fig. 2.

The last solution was used as a standard. The experiment was repeated with different volumes of solution. The results are plotted with the volumes of the solution as abscissæ, and the equivalents of pyridine per iron atom to cause disappearance of the spectrum and colour of reduced hæmatin as ordinates (fig. 2).

It is seen that the curve, as concentration of the solution increases, becomes parallel to the x-axis at two equivalents of pyridine within the limits of experimental error. The result is not effected by alterations in the amount of reducer or by different concentrations of alkali, showing that some essential property of the reduced pigment is in question. Spectroscopic observations were made over a range of pyridine concentrations. No evidence of any third compound could be obtained. In the presence of insufficient pyridine the reduced-hæmatin spectrum was seen together with a pyridine hæmochromogen spectrum; in the presence of ten times the amount of pyridine required to make the hæmochromogen, the two bands were in exactly the same position as in solutions containing less pyridine. Therefore, under the conditions of the above experiment, there appears to be only one pyridine hæmochromogen, and this contains two molecules of pyridine. This is not in agreement with Anson and Mirsky (1925), who brought forward spectroscopic evidence in favour of two hæmochromogens containing different quantities of a nitrogenous compound which they called α- and β-hæmochromogen.

4. *Addition of Pyridine to Reduced Hæmatin in Presence of Carbon Monoxide.*—As was mentioned in a previous section, when pyridine is added to carbon-monoxide hæmatin, the α-band is displaced towards the red end of the spectrum. Then, as an excess of pyridine is added, hæmochromogen makes its appearance and the α-band is displaced towards the violet. The amount of pyridine required to produce the maximum shift towards the red, provided dissociation is negligible, will be a measure of the pyridine involved in chemical combination with CO-reduced hæmatin. A series of solutions of hæmatin in sodium carbonate containing different amounts of pyridine were reduced in the presence of coal gas. The position of the α-band, corresponding to the amount of pyridine present, was measured on a Hartridge reversion spectroscope. Four experiments are given below: Nos. 1 and 2 were carried out in an open vessel, coal gas being bubbled through the solution; Nos. 3 and 4 were carried out in a closed vessel containing coal gas and the solutions vigorously shaken. As before, the pyridine and hæmatin solutions were both 0·032 normal.

Experiment 1.—Hæmatin (0·032 N.), 1 c.c.; 10 per cent. sodium carbonate, 1 c.c.; hydrosulphite, 0·3 gm.; water, 40 c.c.

Cubic centimetres Pyridine per 1 c.c. Htn.	0·7	0·8	0·9	1·0	1·2
Spectroscope reading	2·5	2·1	1·6	1·5	2·6

Experiment 2.—Hæmatin (0·032 N.) 1 c.c.; 10 per cent. sodium carbonate, 1 c.c.; hydrosulphite, 0·3 gm.; water, 40 c.c.

Cubic centimetres Pyridine per 1 c.c. Htn.	0·6	0·8	0·9	1·0	1·1	1·2	1·4
Spectroscope reading	3·2	2·8	2·6	2·3	2·4	2·6	4·4

Experiment 3.—Hæmatin (0·032 N.) 2 c.c.; 10 per cent. sodium carbonate, 3 c.c.; hydrosulphite, 0·3 gm.; water, 100 c.c.

Cubic centimetres Pyridine per 1 c.c. Htn.	0·5	0·7	0·9	1·1	1·3	1·5
Spectroscope reading	5·7	5·3	4·9	4·6	4·4	4·4

Experiment 4.—Hæmatin (0·032 N.) 2 c.c.; 10 per cent. sodium carbonate, 3 c.c.; hydrosulphite, 0·3 gm.; water, 50 c.c.

Cubic centimetres Pyridine per 1 c.c. Htn.	0·5	0·7	0·9	1·1	1·3	1·5
Spectroscope reading	6·0	5·8	5·4	5·3	5·2	5·2

In experiments 1 and 2 it will be noted that there is a minimum reading on the arbitrary scale of the spectroscope at approximately one equivalent of pyridine. In experiments 3 and 4 the reading is nearly constant for amounts of pyridine greater than one equivalent. The change is sharper in experiment 4, where the general concentration is greater and where there is less effect due to dissociation of the complex. The difference in the shift of the α-band in the two sets of experiments is in agreement with the view that both the pyridine and carbon monoxide compete for combination with the pigment. In the first case the supply of carbon monoxide is limited at the moment of formation of the complex, so that hæmochromogen can be formed with the slight excess of pyridine; in the second case, the supply of carbon monoxide is unlimited and no hæmochromogen is formed. Unfortunately, owing to the insoluble nature of the pigment after reduction, no detailed consideration of the effect of the pressures of carbon monoxide on the equilibrium is at present possible.

(5) *Combination with Carbon Monoxide.*—Reduced hæmatin is almost insoluble in water in alkaline solution. But if sodium carbonate is used instead of sodium hydroxide, the precipitation takes place slowly, and can be still further retarded by the addition of gum arabic (Keilin, 1926). The carbon monoxide compounds used in these experiments were prepared by reducing in an atmosphere of carbon monoxide a strong solution of hæmatin in sodium carbonate containing gum arabic. The gum arabic was previously purified by precipitating three times with alcohol containing 1 per cent. hydrochloric acid.

The carbon monoxide compounds prepared in this way were introduced into one of the bulbs of the Barcroft micro-respirometer. Caustic soda was added, the small cup in the centre of the bulb being filled with saturated potassium ferricyanide. The object of the caustic soda is to combine with the CO_2, which would be liberated from the carbonate originally present, owing to the reduction of the ferricyanide. The centre cup contained a small capillary tube which enables the ferricyanide to be readily poured into the bulb when the apparatus is inclined at a suitable angle. In order to avoid undue oxidation by atmospheric oxygen, a stream of coal gas was passed into the bulbs while they were being filled. The other bulb of the apparatus was filled in exactly the same manner with the same reagents minus the hæmatin. Then the apparatus was rapidly exhausted to 40 mm., and twice washed out with the coal gas before finally filling. The method used was that described by Dixon and Tunnicliffe (1923). The taps were then closed after adjusting the pressure in the bulbs to atmospheric. The experiments were performed at room temperature. When, in the usual trough of water, the manometer reading was constant, the ferricyanide was allowed to mix with the fluid in the bulbs. The red colour changed at once to dark brown, and the carbon monoxide was expelled. The manometer was again read, and the evolution of carbon monoxide calculated in the usual way.

Experiment (a).—Carbon Monoxide Hæmatin without Pyridine.

A solution containing 1 c.c. hæmatin 0·032 N., 1·5 c.c. 10 per cent. sodium carbonate, 1 c.c. 1 per cent. gum arabic was reduced in a current of carbon monoxide by the addition of 0·5 c.c. 10 per cent. sodium hydrosulphite. After 10 minutes 2 c.c. were introduced into one of the bulbs of the micro-respirometer. 0·5 c.c. 40 per cent. soda and 0·5 c.c. water were then added. The centre cup contained 0·35 c.c. saturated potassium ferricyanide. The other bulb was filled with the same quantity of reagents with the exception of the hæmatin, which was replaced by an equal volume of water.

Cubic centimetres hæmatin (0·032 N.).	Observed evolution in cubic centimetres.	Calculated for 1 molecule.
0·5	340	358
0·5	290	358
0·33	210	239
0·5	310	358

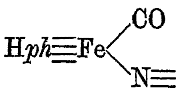
Experiment (b).—Carbon Monoxide Reduced Hæmatin in Presence of Pyridine.

Cubic centimetres original hæmatin solution.	Observed evolution in cubic centimetres.	Calculated for 1 molecule.
0·5	311	358
0·5	352	358
0·5	344	358

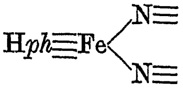
The results obtained with reduced hæmatin are lower than the theoretical. As both reduced hæmatin and the carbon monoxide compound in the absence of pyridine are very insoluble, there is difficulty in reaching the full saturation value for the carbon monoxide. The possibility of there being two molecules of carbon monoxide in combination with the pigment in the absence of pyridine is excluded both by the colour and by the spectrum shown by the fluid experimented on. A mixture of equal parts of uncombined reduced hæmatin and its carbon-monoxide compound would be readily detected by its dull colour and by its spectroscopic appearance.

IV. *General Discussion.*

From quantitative experiments on the combination of pyridine with reduced hæmatin, we have shown above that in CO-hæmochromogen one nitrogen-containing molecule is replaced by CO, there being two such nitrogenous molecules in hæmochromogen itself. By adding the nitrogen compound to CO-reduced hæmatin, one molecule of the former is taken up; by adding excess of the nitrogen compound, the CO is displaced by the taking up of a further molecule of nitrogen compound, and hæmochromogen results. These changes can be observed spectroscopically, and occur when any nitrogen compound is used which gives a typical hæmochromogen, including denatured globin itself. Now, as iron is the only element in combination with the pigment which causes it to have the property of forming hæmochromogen, it is most simple to assume that the nitrogen and carbon monoxide are co-ordinated with the iron directly. The supposition is that the general formula for a CO-hæmochromogen and a hæmochromogen is as follows :—



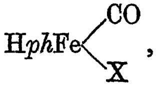
$$\mathrm{H}ph\equiv\mathrm{Fe}\begin{matrix}\diagup\mathrm{CO}\\ \diagdown\mathrm{N}\equiv\end{matrix}$$



$$\mathrm{H}ph\equiv\mathrm{Fe}\begin{matrix}\diagup\mathrm{N}\equiv\\ \diagdown\mathrm{N}\equiv\end{matrix}$$

(where H*ph* is a porphyrin)

and this covers the facts known at present. It also gives the iron atom a co-ordination number of 6, because the pigment itself is only di-chelate and thus can only account for four of the co-valencies of iron. The chief objection to this view is that, in the carbon-monoxide compound of reduced hæmatin, without pyridine or other nitrogen compound, only one molecule of carbon monoxide is present, not two, as might have been expected. However, we could write the formula—



$$\mathrm{H}ph\mathrm{Fe}\left\langle\begin{array}{l}\mathrm{CO}\\ \mathrm{X}\end{array}\right. ,$$

where X is either another molecule of the complex or a molecule of water. There is evidence that reduced hæmatin combines with itself to form large molecules in the absence of the specific reagents in question, because of its insolubility, and the slowness with which it reacts with such reagents.

I hope shortly to publish an account of the dissociation in very dilute solution of pyridine hæmochromogen into pyridine and reduced hæmatin. The dissociation is of a violently " S "-shaped character, resembling a titration curve of a weak base. This shows that either hæmochromogen or reduced hæmatin must be considered highly polymerised, to account for the reaction. On the other hand, the dissociation curve of CO-hæmochromogen into CO-reduced hæmatin and pyridine approximates to a hyperbola, and shows that this reaction is of a comparatively simple type.

It seems that a careful study of the dissociation equilibrium of pyridine and carbon monoxide with the reduced pigment will give information about the nature of the latter. Reduced hæmatin must be regarded as the pigment of hæmoglobin shorn of all its accessory substances, and therefore corresponds to the substance called " hæm " by Anson and Mirsky (1925). The quantitative nature of the chemical reactions of reduced hæmatin in relation both to simple substances and proteins, may eventually make clear the nature of the pigment in hæmoglobin, and explain how it is not irreversibly oxidised by molecular oxygen.

V. *Summary.*

1. The hæmochromogen type of spectrum shown by compounds of hæmatoporphyrin with metals other than iron is not due to the presence of nitrogen compounds. It is suggested that the property of forming hæmochromogens is limited to the iron-porphyrin compounds.

2. Two molecules of pyridine are necessary to convert reduced hæmatin into the corresponding hæmochromogen.

3. Carbon-monoxide hæmochromogen has one molecule of pyridine in combination when produced in the presence of the latter.

4. The carbon-monoxide compounds of reduced hæmatin and pyridine hæmochromogen both contain one molecule of carbon monoxide.

5. On the basis of the experiments with pyridine, and by the parallel changes in spectra produced by other nitrogen compounds, general formulæ for hæmochromogen and its carbon-monoxide compound are proposed.

I am very grateful to Sir Frederick Hopkins for his interest in this work, and to Dr. Keilin for his generous help in connection with spectroscopic measurements.

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Effects of Ca- and K-Ions and Ultra-Violet Rays upon Involuntary Muscles.

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(From the National Institute for Medical Research.)

The antagonistic action of calcium and potassium ions in nutritive solutions has been previously studied by many. According to S. Ringer (1) calcium is necessary in the case of the frog's heart to abolish the toxic action of a physiologically isotonic solution of sodium chloride and maintain the tone of this organ, and potassium controls the action of calcium. S. G. Zondek (2), studying the relationship between the vegetative nervous system and these ions, concluded that the action of sodium and potassium ions is similar to that of the vagus nerve, while the action of calcium corresponds to that of the sympathetic.

The relationship between Ca- and K-ions and ultra-violet rays is of interest. According to Rothman (3) the calcium content of the normal blood is increased when erythema and pigmentation of the human skin results from irradiation with ultra-violet rays. Pincussen (4), investigating this matter, showed that the calcium content is not greatly affected, while that of potassium is decreased; in other words, the ratio of K/Ca decreases after the light bath. It is of interest that adrenalin (a stimulant of sympathetic nerve endings) has the contrary action when injected into animals or human beings; it causes in the blood a decrease of calcium (5) and increase of potassium (6).

The Ca-content of the blood in cases of rickets is remarkably decreased from the normal value (7), but when light baths are given (sunlight, carbon arc or mercury-vapour lamp), it comes back again in a few weeks' time to normal, while recovery from the disease takes place.

In considering the question why light baths act so beneficially in cases of tuberculosis it has been suggested that the ultra-violet rays antagonise an increase of sympathetic tonus, which is supposed to be produced by the toxin of the tubercle bacillus. There is some evidence, then, of an interesting relationship between calcium, potassium and the vegetative nervous system.

L. Hill and Y. Azuma (7) have recently studied the remarkable contraction of involuntary muscles produced by irradiation with ultra-violet rays. In the present research more particularly, the effects are studied of cations

(Ca and K) upon the contraction of the frog's rectum which is produced by irradiation.

The experiments were in two series:—

(1) The rectum was immersed at first in normal Tyrode solution and then changed to one with a certain cation deficiency. After the effect of this had been observed, the rectum was exposed to the mercury-vapour lamp.

(2) The rectum was immersed in a solution which was deficient in certain cations from the beginning.

SERIES I.

The rectum (R) prepared from the winter frog (*R. temporaria*) by the method of J. Schüller (8), was suspended in a bath, which contained 20–30 c.c. of nutritive solution, and fixed to a light lever (L) which recorded the rhythmical movements on a drum (D) (fig. 1). The bath had a quartz plate (Q) on one side which let through ultra-violet rays from a water-cooled mercury-vapour with a lamp quartz window (110 volts, 5 amps.) which was placed at a distance (M) of 3 inches from the rectum. The bath had a small pipe (P) inserted at the bottom to enable change of solution when required. The changing of the solution was done very slowly, so as to give the rectum as little mechanical irritation as possible. A single or double sheet of thick glass (3 mm.) was used to cut off the shorter wave-lengths of the ultra-violet rays when the effect of the near ultra-violet and visible rays was tested. Oxygen (O_2) was bubbled through the solution, as this is favourable to orderly rhythmical contraction.

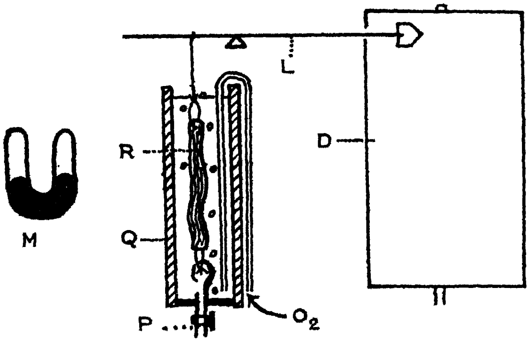

FIG. 1.

(1) *Normal Tyrode Solution used.**

Changing the solution irritates the rectum only a little, and after a couple of bigger rhythmical contractions it shows again normal quiet regular movements. Rays from the mercury-vapour lamp which pass through window glass have no effect on the normal rectum, but when it is exposed to rays which pass through blue uviol-glass and contain shorter wave-lengths of ultra-violet rays, there is a slight increase in tonus as well as frequency. When the rays from the M.V.L. irradiate the rectum without any screen, there occurs very remarkable increase in tonus and frequency of its movements. The height of each rhythmical contraction overtops the base-line, which is already raised by the increased tonus (figs. 2 and 3).

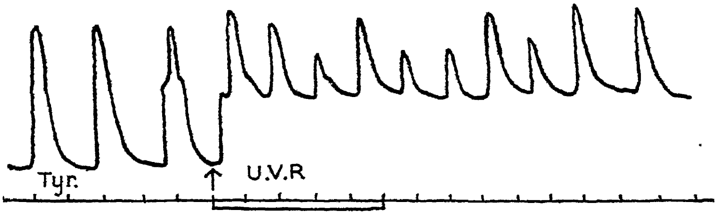

FIG. 2.

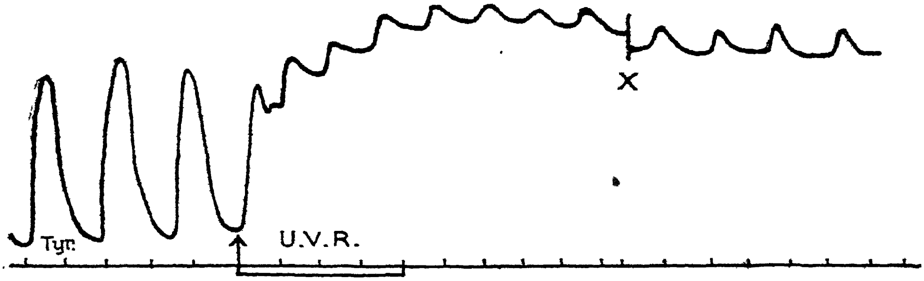

FIG. 3.

The normal tonus and rhythmical contraction may return when the rectum is not too much exposed (say, for 10 minutes), but with longer exposure it ceases to contract rhythmically and stops in a state of tonus.

(2) *Na-Ions in Solution.*

When the Tyrode solution is changed for one in which NaCl is retained and $CaCl_2$, KCl and $MgCl_2$ left out, the tonus comes down gradually after a sudden initial increase. The frequency, too, increases. Exposure to the naked M.V.L. gives then only a very slight increase in tonus, and rhythmical movements come to stop earlier than in the case of the normal solution (fig. 4).

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* (I) NaCl, 0·65; KCl, 0·02; $CaCl_2$, 0·02; $MgCl_2$, 0·01; H_2O, 80.
(II) $NaHCO_3$, 0·1; NaH_2PO_4, 0·005; H_2O, 20.
(I) and (II) are mixed just before use.

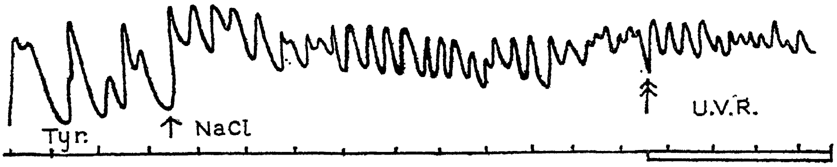

FIG. 4.

(3) *Na- and K-Ions in Solution.*

In the next solution tried $CaCl_2$ and $MgCl_2$ were left out and KCl and NaCl retained. The rectum exposed in this solution to the naked M.V.L. showed an increase in tonus (fig. 5) smaller than that obtained in the next case.

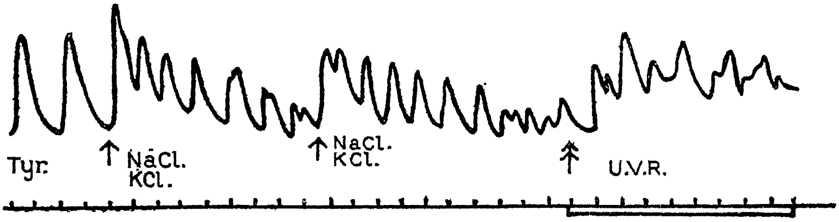

FIG. 5.

(4) *Na- and Ca-Ions in Solution.*

The solution contained no KCl and $MgCl_2$, but Ca-ions besides Na-ions were present in the same proportion as in normal Tyrode solution. After a sudden increase of tonus with more frequent rhythmical contraction, the tonus lessened sharply and the movements became less and less: these changes were reversible if the rectum was returned into the normal solution in proper time. As a rule, rays through glass had no effect, but rays from the naked M.V.L. gave always a remarkable tonus increase and sometimes the rectum lost its rhythm and stayed in a tetanic state (fig. 6). Reversion was possible. Recovery of rhythm was seen after addition of KCl.

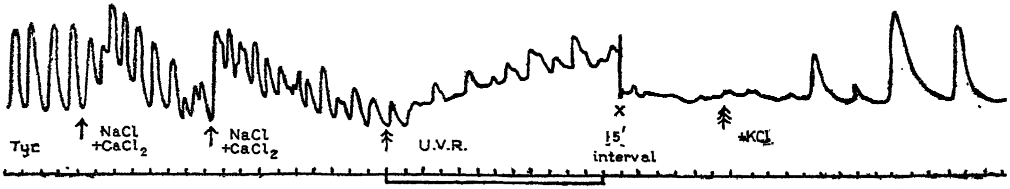

FIG. 6.

(5) *Na- and Mg-Ions in Solution.*

In a solution with no $CaCl_2$ and KCl but containing $MgCl_2$ besides NaCl, the rectum lost its contractility on radiation with the M.V.L. and stopped in a relaxed state—just the reverse to the results obtained with Ca-ions (fig. 7).

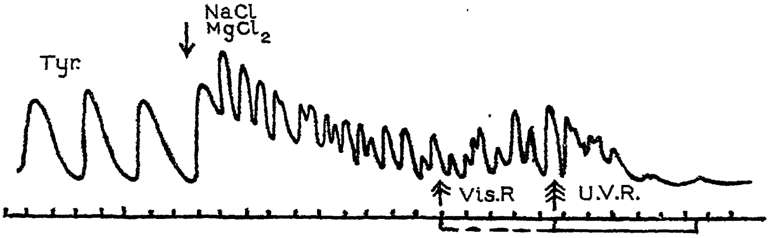

FIG. 7.

(6) *Na-, K- and Ca-Ions in Solution.*

$MgCl_2$ in the normal solution appeared to be of small account; if it was left out, the rectum behaved itself as in the normal solution.

(7) *Na-, K- and Mg-Ions in Solution.*

In this case, the results were nearly the same as with Na- and K-ions (fig. 8).

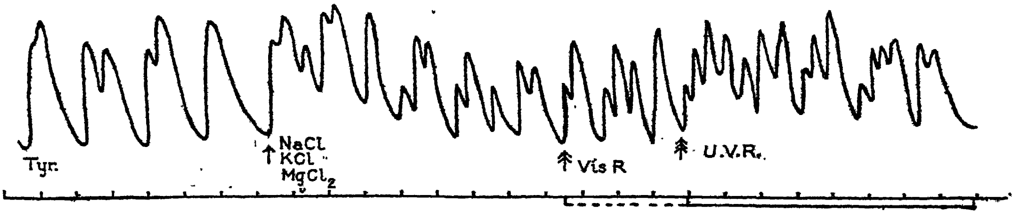

FIG. 8.

(8) *Na-, Ca- and Mg-Ions in Solution.*

It was of interest that the movements of the rectum in a solution, which contained Ca- and Mg-ions besides Na-ions, showed no noticeable changes either in tonus or in rhythm on irradiation. Contraction gradually lessened and finally ceased. This suggests some antagonistic action between Ca- and Mg-ions.

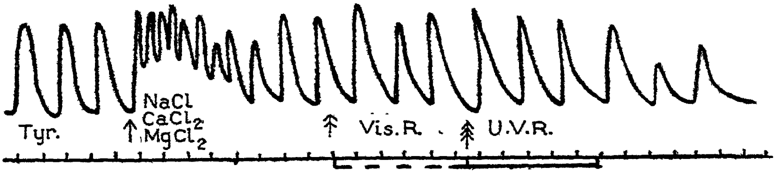

FIG. 9.

SERIES II.

There were some alterations in method in this series of experiments ; a silica plate of 2 mm. thickness was used instead of a quartz one. Ultra-violet rays passed through this well, as was shown by testing the killing-time of Infusoria. An air-cooled M.V.L. (110 volts, 5 amps.) was employed instead of a water-cooled one, and by means of irrigation of tap water along the outside of the silica plate, it was possible to avoid almost completely the heating effect of the M.V.L.

The following was prepared as a fundamental solution, to which certain salts (KCl, $CaCl_2$) were added when they were required :—

NaCl, 0·65 ; NaH_2PO_4, 0·005 ; $NaHCO_3$, 0·1 ; H_2O, 100.

(1) *Na-Ions in Solution.*

When the rectum was suspended in the fundamental solution, which contained neither K- nor Ca-ions, but only Na-ions, the tonus increased gradually and rhythmical movements began to appear in a few minutes and reached a maximum in about 10 minutes. Then the tonus decreased, and after 2 hours it was just possible to notice that the rectum was contracting rhythmically. If it was now exposed to the M.V.L., the tonus increased a little but not remarkably (fig. 10). If, however, the irradiation was done when the movements reached their maximum, the tonus increase was rather large and not very different from that in the normal Tyrode solution (fig. 11).

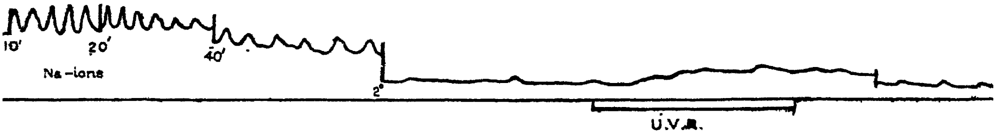

FIG. 10.

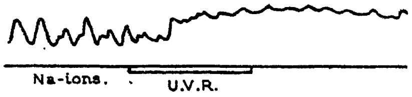

FIG. 11.

(2) *K-Ions in Solution.*

The concentration of KCl was made as in the normal solution (0·02 per cent.). Tonus then increased and rhythmical movements appeared in a few minutes. After 5 hours there were still rather big contractions, although the frequency was getting much less and the tonus decreasing little by little. When exposed to ultra-violet rays, the tonus as well as the rhythm recovered a little (fig. 12). The rectum when irradiated shortly after its suspension in this solution showed as big a tonic contraction as that in the normal Tyrode solution (fig. 13).

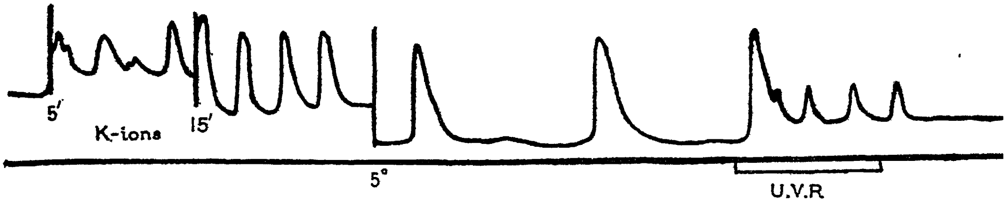

FIG. 12.

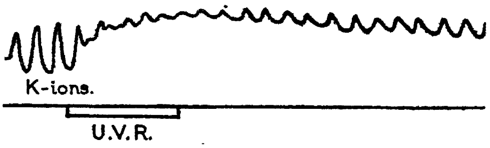

FIG. 13.

(3) *Ca-Ions in Solution.*

When calcium was added as in the normal solution (0·02 per cent. $CaCl_2$), the rectum began to move in a few minutes rhythmically and beautifully. The tonus increased higher and higher and the contraction then became gradually weaker. After 2 hours, the contractions stopped although the tonus kept still increasing. If, then, the rectum was irradiated with the M.V.L., the tonus increased still more (fig. 14). If the rectum was exposed to the lamp shortly after immersion and beginning to contract, the tonus increase was like that in the normal solution (fig. 15).

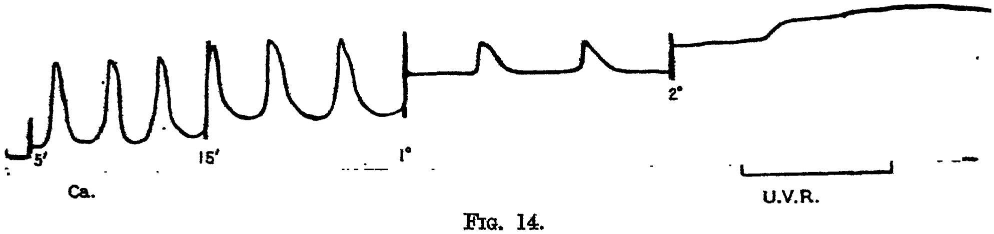

FIG. 14.

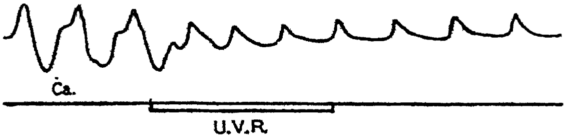

FIG. 15.

Discussion.

Bearing in mind that ultra-violet rays have a great influence on the calcium metabolism of animals as well as of man, it is interesting to find that these rays act most effectively on involuntary muscles when calcium chloride is present in the nutritive solution.

The rectum suspended in normal Tyrode solution still shows after 4 hours big rhythmical contractions. The rhythmical movement ceases first in the "fundamental" solution *plus* $CaCl_2$, and last in this solution *plus* KCl, and in intermediate time in this solution *plus* Na-ions. The Ca-ion has the power to increase tonus, but the K- and Na-ions have not.

In nearly every experiment the rectum contracts more or less on exposure to irradiation with the M.V.L. Although there may be no Ca in the nutritive solution at the beginning of the experiment, there is still in the tissue some calcium, both combined and uncombined, which may contribute to the contraction when irradiated. Those ions which the nutritive solution lacks will diffuse out into the solution the longer the rectum is kept in such solution. Hence the contraction fails as the calcium diffuses out.

Conclusions.

(1) By leaving out certain constituents of the normal Tyrode solution, rhythmical contractions of the frog's rectum are affected in regard to tonus, amplitude of contraction and frequency. In each case tonus and frequency are somewhat increased at first, when the solution is changed, tonus then comes down gradually and amplitude gets less than normal.

(2) Rays from a mercury-vapour lamp through glass have no effect in almost all cases.

(3) The effects of shorter wave-lengths of the ultra-violet rays are very different with different solutions.

(4) The rectum of the frog in isotonic solution of sodium chloride loses its rhythmical contraction as well as its tonus in one hour. When in such a state it is exposed to the M.V.L., only a small increase of contraction is noticeable.

(5) When to this solution K-ions are added, nearly the same results are obtained, but the rectum is able to keep its rhythmical contraction much longer. The increase of tonus on irradiation is as small as in (1).

(6) In an isotonic sodium chloride solution with Ca-ions added, the rectum behaves quite differently; its tonus goes on increasing, while its rhythmical contraction ceases in a short time (half an hour). If after one hour it be

irradiated with ultra-violet rays, it contracts still more. Calcium is connected with the increase of tone through irradiation.

(7) When in addition to sodium chloride the solution contains only a small percentage of magnesium chloride, exposure to the ultra-violet rays causes relaxation of tone and disappearance of rhythm. When, in addition to sodium, calcium and magnesium ions are present, in the proportions of their occurrence in Tyrode solution, but no potassium ions, ultra-violet radiation does not increase tonus or alter rhythm, but on prolonged exposure of the rectum to this the height of contraction diminishes and contraction finally ceases.

I wish to thank Prof. L. Hill and Dr. H. H. Dale for their valuable suggestions and criticism.

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Recovery from Vigorous Exercise of Short Duration.

By R. M. SARGENT, B.Sc.[*]

(Communicated by Prof. A. V. Hill, F.R.S.—Received October 25, 1926.)

(From the Department of Physiology and Biochemistry, University College, London.)

In the experiments described below an endeavour has been made (*a*) to follow the course of recovery from vigorous exercise of short duration, (*b*) to determine whether it be possible to arrive at some average figure which adequately represents the degree of recovery of different subjects at any time after the end of exercise, and (*c*) to investigate the magnitude and direction of any alteration that may occur in the basal metabolism as a result of the muscular work performed. Attention was confined to exercise such as described above, as sufficient data are available in the previous work of A. V. Hill[†] for following the course of the recovery process after prolonged exercise of a similar nature.

Procedure.

The object of the experiments was to determine the total amount of oxygen used during successive time-periods from the end of exercise, *i.e.*, to follow the rate at which the oxygen debt incurred in the exercise was " paid off " during the recovery period.

At the beginning of each experiment, the subject lay on a couch and rested for at least 30 minutes, after which a collection of his expired air was made for 10 minutes: this collection served as a " basal " for that particular experiment. During the foreperiod of rest the subject breathed through the mouthpiece, as it was found in the preliminary experiments that unless this precaution was adopted the respiration of the subject was altered to an appreciable extent immediately he took the mouthpiece into his mouth, and that the analyses of the resting collection obtained under such conditions gave a respiratory quotient much below the normally accepted value.

The subject then did " standing-running " at a certain speed, for either half a minute or one minute, after which he lay down on the couch ; collection of his expired air was started immediately on the cessation of the exercise and was continued in most cases for 70 minutes. Collection was made in a 600-litre tank, from which samples were withdrawn at successive intervals during the recovery period. The methods of stirring the expired air, and of recording the ventilation-volume and the time of sampling, were similar to those adopted by Furusawa.* The samples were subsequently analysed on an automatically operated Haldane apparatus. In all, 12 experiments were completed on four different subjects and over 200 analyses made.

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[*] Working for the Industrial Fatigue Board of the Medical Research Council.

[†] Hill, Long and Lupton, 'Roy. Soc. Proc.,' B, vol. 97, p. 104 (1924).

Calculation of Results.

The *total* amount of oxygen used, from the end of the exercise to the time of sampling, was calculated from the analysis and volume and plotted on a graph against the time of recovery.

Fig. 1 gives the graph so obtained for Experiment S2 ; as will be seen from the curve, the total amount of oxygen used increases rapidly during the firs

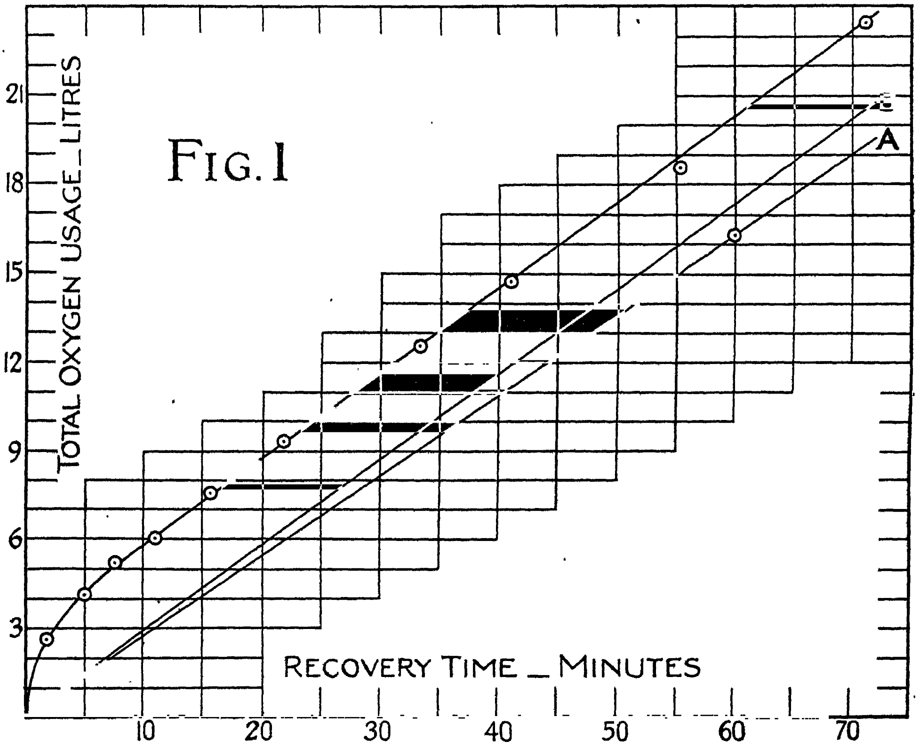

FIG. 1.

Total Recovery Oxygen-Usage Curve for Exp. S2. Curve A represents the resting oxygen consumption before exercise, and curve B (the base-line referred to in the text) the resting oxygen usage after the recovery period.

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* Furusawa, 'Roy. Soc. Proc.,' B, vol. 99, p. 148 (1926).

10 minutes after the end of exercise and ultimately becomes a linear function of the time of collection. It is obvious that in this latter portion of the curve the oxygen intake of the subject has reached its post-exercise resting level.

In all experiments the first four or five points lie well on the curve and little smoothing is required; the subsequent points generally show some small displacement, the difference, however, between the observed and the "smoothed" values lying well within the limits of experimental error permissible in observations of this nature.

The post-exercise resting oxygen intake can readily be determined by drawing a straight line (the "base-line") on the graph, through the origin and parallel to the linear portion of the curve, the distance between this base-line and the straight part of the curve representing the total excess oxygen used over the resting value (the oxygen debt) during the recovery-period. The total amount of oxygen used up to any definite time after the end of exercise can be determined in a similar manner by taking the distance between the base-line and the curve at the particular time under consideration.

Determination of the Percentage Recovery.

By "percentage recovery" at any time after the end of exercise is meant the amount of excess oxygen, used during the recovery-period up to that particular time, expressed as a percentage of the total oxygen to complete recovery (see Table I).

Discussion.

The rate of recovery from vigorous exercise of short duration in healthy young male subjects is exceedingly rapid, especially during the first 10 minutes after the end of exercise; it then gradually diminishes until recovery is complete. Recovery from exercise such as is here described is completed in 20 to 25 minutes, though after the first 10 minutes of the post-exercise period the subjects are, on an average, nine-tenths recovered.

The similarity of the course of recovery for different subjects, from exercise of the same nature and not differing too much in severity, receives confirmation in the previous work of A. V. Hill,* who found that after severe and prolonged "standing-running" recovery was complete in all cases in 80 minutes after the end of exercise.

The division of the recovery process into two phases, viz., a short period of rapid recovery followed by one in which the recovery process "tails off," has been explained by A. V. Hill (*loc. cit.*, p. 108) as being due to the passage of lactic

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* Hill, Long and Lupton, 'Roy. Soc. Proc.,' B, vol. 97, p. 104 (1924).

Table I.—Percentage Recovery at Various Times after the End of Exercise.

Details of Experiments.

The letter prefixed to the number of each experiment indicates the subject :—

Subject H.—J. P. H. Age 24 years ; weight 56 kilos ; cross-country runner.
,, C.—A. W. C. Age 35 years ; weight 54 kilos ; ex-Regular soldier.
,, S.—B. S. Age 22 years ; weight 70 kilos ; exceedingly powerful and an excellent swimmer.
,, P.—J. R. P. Age 23 years ; weight 59 kilos ; athletic.

The exercise for each experiment is as follows :—

Experiment 1.—" Standing-running " at 202 steps per min. for 1 minute.
,, 2.— ,, ,, 248 ,, ,, 30 seconds.
,, 3.— ,, ,, 248 ,, ,, 1 minute.
,, 4.— ,, ,, " all-out " for 30 seconds.

Time from end of exercise.	2½ mins.	5 mins.	7½ mins.	10 mins.	15 mins.	20 mins.	25 mins.
Percentage recovery—							
Experiment H1	70·0	88·9	96·7	99·5	100·0	100·0	100·0
,, H2	81·1	96·8	98·8	100·0	100·0	100·0	100·0
,, H3	74·5	86·3	93·0	96·5	100·0	100·0	100·0
,, H4	51·7	72·0	84·2	90·7	98·8	100·0	100·0
Average for H	69·3	86·0	93·2	96·7	99·7	100·0	100·0
Experiment C1	48·6	64·8	75·0	81·5	90·9	97·1	100·0
,, C2	82·8	90·6	93·8	97·2	100·0	100·0	100·0
,, C3	72·5	88·0	95·5	99·7	100·0	100·0	100·0
,, C4*a*	64·5	82·2	92·2	97·4	100·0	100·0	100·0
,, C4*b*	46·9	73·0	84·4	90·7	98·0	100·0	100·0
Average for C	63·1	79·7	88·2	93·3	97·8	99·4	100·0
Experiment P1	49·2	69·9	83·5	90·9	98·7	100·0	100·0
Experiment S1	85·5	98·7	100·0	100·0	100·0	100·0	100·0
,, S2	77·6	92·8	98·4	100·0	100·0	100·0	100·0
Average for S	81·6	95·8	99·2	100·0	100·0	100·0	100·0
Average for all subjects	67·1	83·7	91·3	95·3	98·9	99·8	100·0

acid from the active muscles into the blood-stream and its diffusion into other tissues of the body, in which its oxidative removal is prolonged. Thus the first phase of the recovery process is the oxidative removal of lactic acid at its site of formation, while the second and more prolonged phase represents its removal in a similar manner from the other tissues.

The rate of recovery varies with the subject and with the vigour of the exercise : the more prolonged and the more vigorous the exercise, the longer is the subsequent recovery-period, different subjects exhibiting a marked variability in their recovery-time from exercise of the same magnitude. Of the four subjects examined, S, who is a well-built young man and an excellent swimmer, exhibits the most rapid recovery, while C, who is more elderly, shows the longest period for recovery. Fig. 2 gives the average percentage-recovery curves for subjects C, H and S; curve 4 of this figure (average percentage-recovery for all subjects) includes that for subject P, on whom only one experiment was performed.

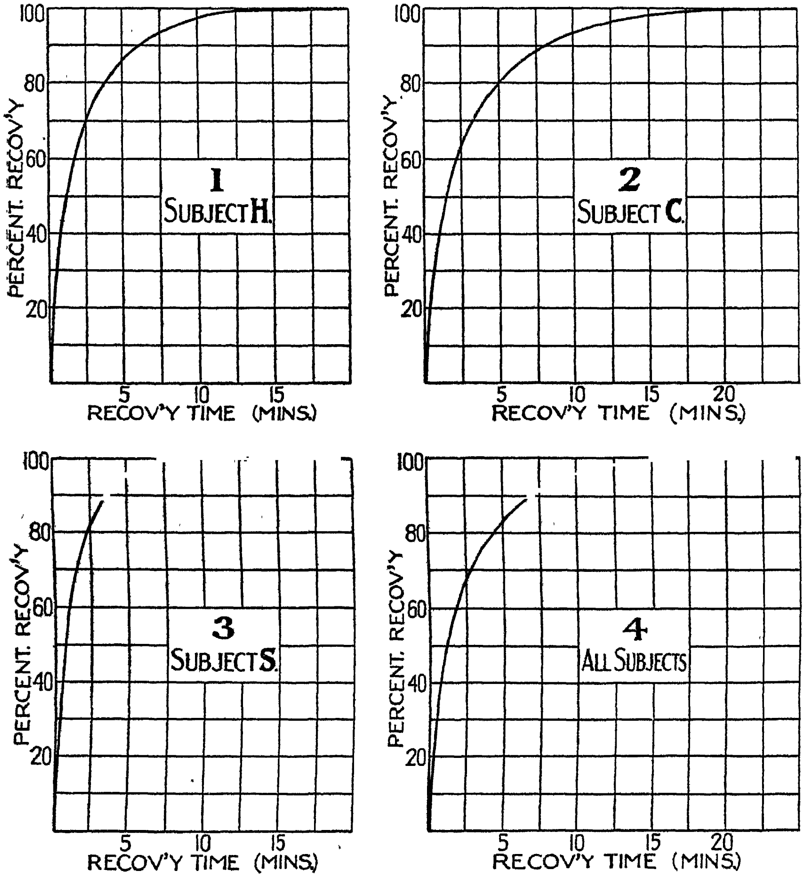

FIG. 2.—Average Percentage-Recovery Curves.

Curve 1—Average of four experiments.
Curve 2—Average of five experiments.
Curve 3—Average of two experiments.
Curve 4—Average for all four subjects.
(For details of experiments, see Table I.)

When a large number of observations have to be made, it is extremely inconvenient if the total oxygen usage has to be determined in each experiment to the end of the completed recovery process. Attempts, therefore, have been made to avoid the necessity of such a proceeding by determining the oxygen usage up to some pre-determined stage, say 10 minutes, of recovery, the total excess recovery oxygen being reduced from the value so obtained by the application of some previously determined correction. The average percentage-recovery figures given in Table I have been applied to the calculation of the oxygen debts, on the assumption that after 10 minutes of recovery always the same fraction of the oxygen debt has been "paid off." The total excess oxygen usage up to 10 minutes from the end of exercise, as actually observed, was divided by the degree of recovery as obtained from the average percentage-recovery at 10 minutes, and the result was compared with the value of the oxygen debt as actually determined up to the end of complete recovery. The error in the oxygen debts so calculated was found to be, on an average, *minus* 0·2 per cent., that for H being *plus* 0·5 per cent., and for C approximately *minus* 1·0 per cent. No great weight, however, can be laid on this comparatively good agreement, the important question being how far *individual* observations agree with *individual* calculations made in this way.

The figures of Table I show clearly that the recovery processes are not the same in different subjects and in the same subject after exercise of differing severity, so that a method of determining the oxygen debt incurred by the use of an average recovery curve is obviously inaccurate; the error involved is much greater than the average figures given above would indicate, for on an examination of the oxygen debt so calculated for each experiment, it was found that the percentage error ranged from *minus* 14·7 per cent. (Experiment C1) to *plus* 4·9 per cent. (Experiments H2 and S1).

The recovery curves are all similar in a general way, and although they cannot be used as *exact* average curves when quantitative calculations have to be made, information of a general nature can be obtained from them. For example, the average recovery curve that can be derived in the manner indicated above may be used to calculate how long an interval should be allowed between the different events in a sports meeting in which the same competitor is running, or to specify how long a labourer doing some exhausting work of short duration should be given to recover from it.

Alterations in the Basal Oxygen Consumption during the Recovery Period.

In the application of the method described above for following the oxygen usage during the recovery period, it was found that an alteration occurs in the basal metabolism of the subject, in so far as the oxygen consumption after exercise does not return exactly to the basal level exhibited before exercise. The method adopted of determining the "basal" oxygen consumption after exercise, which involves the consideration of at least six separate observations and samplings of which an average is taken, appears to place the accuracy of this observation beyond all question. The differences between the oxygen consumption before and after exercise are given in Table II.

Table II.—Percentage Change in the "Basal" Oxygen Consumption as a Result of Exercise.

Experiment.	H1	H2	H3	H4	C1	C3	C4*a*	C4*b*	P1	S1	S2
Basal oxygen consumption, cubic centimetres per minute—											
(*a*) Before exercise	263	267	223	256	215	221	236	192	281	250	272
(*b*) After exercise	259	255	256	260	244	231	268	212	283	301	289
Percentage change following exercise	−1·5	−4·5	+14·8	+1·6	+13·5	+4·5	+13·5	+10·4	+0·7	+20·4	+6·3

In two experiments, H1 and H2, a small fall in the oxygen consumption was observed, while in the remainder the exercise caused a definite increase in the "basal" oxygen usage. The alterations in the metabolism lie between *minus* 4·5 per cent. (Experiment H2) and *plus* 20·4 per cent. (Experiment S1), with an average of approximately *plus* 7 per cent. Hill, Long and Lupton[*] found that after severe exercise the resting oxygen intake of their subjects returned to a constant level also some 7 per cent. higher, on an average, than it was before the exercise. The change, though small, is quite definite, and in view of the method adopted above in the determination of the resting oxygen consumption after exercise, together with its appearance to the same extent after exercise differing greatly in severity and in duration, it cannot be regarded as being in any way an integral part of the recovery process. In all experiments the metabolism of the subject has clearly attained to a constant level, and any alteration in the basal levels can only result from a genuine change in the metabolism (*e.g.*, of the circulatory and respiratory systems) caused by the exercise.

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[*] 'Roy. Soc. Proc.,' B, vol. 97, p. 105 (1924).

Summary.

(1) Recovery from vigorous exercise of short duration is extremely rapid, especially in the first 10 minutes immediately after the cessation of exercise. Average recovery curves are given for several subjects.

(2) The rate of recovery varies somewhat with the subject and with the severity and duration of the exercise. It is impossible, therefore, to arrive at any average figure which will adequately represent the percentage-recovery at any time after the end of exercise for different subjects and for exercise of differing severity.

(3) It is inadvisable, therefore, in determining the total recovery oxygen after exercise, to measure the amount used during a period of incomplete recovery and to apply a correction.

(4) As a result of the exercise the oxygen consumption at rest was found to be increased, on the average, by about 7 per cent. This change appears to result from a genuine alteration in the level of metabolism and cannot be regarded as an integral part of the recovery process as such.

I wish to express my sincere thanks to Prof. A. V. Hill for the help and advice that he has so kindly given me during the performance of these experiments. I desire also to acknowledge my indebtedness to the Medical Research Council for a personal grant which enabled me to undertake this work, and for a grant to cover the expenses incurred.

Reflex Fractionation of a Muscle.

By SYBIL COOPER, D. E. DENNY-BROWN and Sir CHARLES SHERRINGTON, O.M., F.R.S.

(Received November 17, 1926.)

(From the Physiological Laboratory, Oxford.)

Studying spinal reflexes Camis (1) (1910) from observations on *M. semitendinosus* (cat) reached the conclusions that "the cells of a spinal motor centre can be regarded from a functional point of view as divided into several independent groups," but that "such independence is however not absolute." The present experiments pursue a like inquiry. That in a reflex evoked by *weak* excitation of the afferent nerve the resulting contraction of the muscle may involve a portion only of the muscle has common acceptance. Camis's observations, however, employed *maximal* stimuli and yet the muscle evidenced fractional responses; whereas later (3) Dreyer and one of us found, contrary to previous (4) experience, that reflex tetani in some instances activated the sum-total of the muscle. Both of these observations are confirmed by the present experiments.

Method.

The sample muscles taken have been a hip-flexor, *tensor fasciæ femoris*, a knee flexor, *semitendinosus*, and an ankle flexor, *tibialis anticus*. The preparation (cat) has been spinal, with cord transection, performed under deep anæsthesia, in the anterior lumbar region, prior to intercollicular decerebration, the anæsthesia being later relaxed. All other muscles of the limb except that one attached to the myograph have been paralysed by resection or nerve-section. The limb has been securely fixed by steel drills clamped to the table. For exciting reflex contraction various afferent nerves, as cited below, of the ipsilateral limb have been stimulated by faradisation. The inductorium has been coreless and its primary circuit fed by a current of less than 0·2 amp. An optically recording myograph with isometric registration and of the pattern described in a previous paper (2) has been used.

A reason for choosing flexor rather than extensor reflexes for the present observations has been that the spinal contraction responses of the former are of the "*d'emblée*" type (5), the climax of the contraction being reached early in the response. The fullest tension developed by the reflex was thus obtainable by relatively brief stimuli, whereas with recruiting reflexes (5), the culmination of the recruitment would be required, involving much longer stimulations with the attendant risk of "fatigue," polarisation, etc. In our statement of results the tension-value stated for a reflex is that of the climax of the contraction.

The reflex contractions of *d'emblée* type yield often a myogram of jet-like form, with high initial climax, followed by rapid trailing decline. The high initial peak is, like the onset of spinal flexor reflexes in general, rapidly developed, often more rapidly than is the ascent to the plateau of motor-nerve tetani of similar tension under faradisation of like frequency. A recording system tends to exaggerate such a peak. The myograph employed gave, however, good evidence of full avoidance of this defect in the most extreme demands made upon it. Thus in plateau contractions, the ascent to which developed more than 4 kilo. tension in 0·04 sec., and gave an excursion of more than 8 cm. on the record, the records consistently showed no trace of overshoot of the plateau.

An object of the present inquiry has been to ascertain how far the individual afferent nerve under *maximal* stimulation activates the given muscle in its entirety or only partially, in other words activates its sum total of motoneurones or but some fractional proportion of them. For judging whether a given reflex contracts the whole or only part of the muscle the tension developed by it has been measured in the same experiment against either or both of the following two standards: (1) the greatest reflex tension obtained, a tension lower than that being taken to mean partial contraction only; (2) the tension developed by maximal tetanus under similar but direct stimulation of the motor nerve with shock-series of strength and frequency sufficient to ensure maximal character for the tetanus (3). This direct stimulation of the motor nerve was in a number of experiments not made until after severance of that nerve, at the end, therefore, of that experiment. There is then, however, risk of several disturbing contingencies. The circulation in the fixated muscle may have deteriorated; the muscle may have become somewhat fatigued by the preceding reflex contractions; it is not easy to preclude alteration in initial tension due to the nerve-severance, or to be sure that other disturbance from the fresh nerve-section has subsided at the time of the direct stimulation. We have therefore additionally, in a number of experiments, applied direct maximal stimulation to the intact nerve of the muscle at intervals during the course of the experiment.

That this procedure inflicted no damage was consistently shown by unimpairment of the subsequent reflexes. The myograms so obtained were of the "peripheral" tetanus type, except that the later part of the terminal relaxation from the plateau was prolonged by some "after-discharge" contraction, as was to be expected. These controls were serviceable for judging deficiency in the final cut-nerve tetani. The "maximal peripheral tetanus" standard allows, we assume, assessment of a reflex contraction from the same experiment in terms of the contraction of the total muscle. The other standard, *i.e.*, the muscle's strongest reflex contraction in the experiment, although showing that contractions less powerful than it are fractional, does not, unless the largest reflex be definitely shown to activate the whole muscle, express the size of the smaller reflex contraction as a fraction of that of the entire muscle. In light of the all-or-none principle of reaction of the motor units of the muscle, explaining the grading of muscle contraction as their additive result, the fractions read by the above standards should indicate relative quantities of "motor units" reacting in the compared contractions, by "motor units" (8) being understood the axon of a motoneurone together with the muscle fibres it innervates.

Results.

In regard to *maximal* stimulations of the respective afferent nerves results reached have been the following :—

§ (1) Judging by the "maximal peripheral tetanus" standard, instances have been rare where a reflex evoked by any single afferent nerve, among those examined, has caused contraction of the whole of the test-muscle. This renders it the more significant that single-shock spinal reflexes of these flexor muscles should not infrequently develop tensions exceeding that of the maximal "motor-nerve" twitch of the muscle (12). The smaller afferent nerves have without exception activated the muscle partially only.

Examples (*stimuli maximal*) :—

	Afferent nerve.	Gm. tension of reflex tetanus / of max. mot. tet.	Reflex as percentage of max. mot. tet.
M. tensor fasciæ femoris—			
	Internal saphenous	$\frac{1300}{1760}$	74
	,, ,, ...	$\frac{2700}{4300}$	63
	N. of quadric extensor ..	$\frac{1250}{1760}$	71
	Femoralis nerve	$\frac{1600}{1760}$	91

Examples—continued.

Afferent nerve.	Gm. tension $\frac{\text{of reflex tetanus}}{\text{of max. mot. tet.}}$	Reflex as percentage of max. mot. tet.
M. tensor fasciæ femoris—		
Peroneal..	$\frac{1300}{1760}$	74
,,	$\frac{2850}{4300}$	67
Popliteal	$\frac{1200}{4300}$	28
M. semitendinosus—		
Internal saphenous in thigh	$\frac{1900}{2990}$	63
N. of sartorius	$\frac{1500}{2990}$	50
N. of quadriceps and sartorius	$\frac{2740}{2990}$	91
Superficial obturator	$\frac{1270}{2990}$	42
External cutaneous of groin	$\frac{830}{2990}$	28
Internal plantar	$\frac{850}{3000}$	28
External plantar	$\frac{950}{3000}$	32
Popliteal	$\frac{1550}{2420}$	64
,,	$\frac{2630}{2990}$	87
A dorsal nerve to digital cleft..	$\frac{1380}{2420}$	57
Another dorsal nerve to digital cleft	$\frac{2540}{2990}$	84
Peroneal..	$\frac{1600}{1600}$	100
,,	$\frac{2970}{2990}$	99

Examples—continned.

	Afferent nerve.	Gm. tension of reflex tetanus / of max. mot. tet.	Reflex as percentage of max. mot. tet.
M. semitendinosus—			
	Peroneal..	$\frac{2560}{2420}$*	>100
	,, ...	$\frac{2950}{3000}$	98
	,, ...	$\frac{3190}{3180}$	100·5
	Small sciatic	$\frac{1860}{2990}$	62
M. tibialis anticus :—			
	Internal saphenous in thigh ..	$\frac{800}{2460}$	32
	External cutaneous of groin ..	$\frac{1060}{2860}$	37
	Superficial obturator	$\frac{165}{2460}$	6·7
	Deep obturator	$\frac{400}{2460}$	16
	N. of quadriceps and sartorius	$\frac{1190}{2460}$	44
	A dorsal nerve of digital cleft ..	$\frac{890}{2460}$	32
	Musculo-cutaneous division of peroneal	$\frac{1700}{2460}$	69
	,, ,, ,,	$\frac{1480}{2200}$	67
	Small sciatic	$\frac{1320}{2860}$	45
	,, ,, ...	$\frac{700}{1190}$	58
	Internal plantar	$\frac{930}{1900}$	49
	,, ,, ...	$\frac{1240}{2460}$	50

* Muscle somewhat run down at end of experiment.

Examples—continued.

Afferent nerve.	Gm. tension. of reflex tetanus / of max. mot. tet.	Reflex as percentage of max. mot. tet.
M. tibialis anticus—		
Internal plantar	$\frac{1160}{2100}$	55
" "	$\frac{1440}{2200}$	65·5
" "	$\frac{920}{1190}$	77
" "	$\frac{1800}{2350}$	78
External plantar	$\frac{900}{1900}$	47
" "	$\frac{1240}{2460}$	50
" "	$\frac{1290}{2100}$	61
" "	$\frac{1690}{2350}$	72
N. of post tibial muscles . .	$\frac{1525}{2350}$	65
N. of sural triceps [external division]	$\frac{370}{1190}$	30
" " "	$\frac{800}{2350}$	34
" " [internal division]	$\frac{850}{2350}$	38
Popliteal	$\frac{1740}{2460}$	70
"	$\frac{1820}{2350}$	78
"	$\frac{2300}{2860}$	80
"	$\frac{1550}{1900}$	81
Hamstring	$\frac{1130}{2100}$	54
"	$\frac{1800}{2860}$	63

§ (2) Speaking generally, the fractional contraction in the test-muscle is larger for the larger afferent nerve than for the smaller. This rule, however, in application to its respective test-muscle is subject to modification by another (9), namely that, broadly taken, an afferent nerve segmentally more remote from that muscle tends to activate it less than does a nerve otherwise comparable but less remote segmentally. Thus: Popliteal is a larger afferent nerve than is external cutaneous of groin, but the latter, segmentally nearer to *M. tensor fasciæ femoris*, contracts that muscle the more powerfully. Further, besides complication by segmental relationship, the general rule seems subject to a proviso that certain quite small afferent nerves provoke in a respective test-muscle reflexes specifically large. Thus, for instance, two of the small dorsal digital nerves of the foot taken near their digital clefts have evoked reflexes of 80 per cent. and 84 per cent. respectively in semitendinosus; and the external and internal plantar nerves in the foot have given reflexes of 77 per cent. and 89 per cent. respectively in tibialis anticus.

A limitation to the general rule put forward is met with when, as rarely happens, a large collection of afferent fibres gives a reflex which engages the entire test-muscle. The reflex contraction being then already maximal, the stimulation of yet additional afferent nerve-fibres increases its height no further. Thus: with semitendinosus muscle, musculo-cutaneous division of peroneal evoked a reflex of 3000 gm. tension: the whole peroneal evoked similarly 3000 gm., and the maximal tetanus by stimulation of motor nerve similarly gave 3000 gm. Nevertheless, that with increase of size of the afferent nerve there goes increase in size of the reflex is broadly apparent. This argues that the numerical strength of a collection of afferent fibres is an important determinant of the size of the reflex effect. It indicates that the additive principle obtains on the afferent side as well as on the efferent. Exemplifying this further is that, as often seen in our experiments, a parent afferent nerve evokes a reflex larger than does any one of its tributary branches (thus: table in § 7, below) though the excess may be small, and under fluctuation of the reflexes is sometimes not demonstrable in a particular experiment and instance.

§ (3) The size of the fraction of test-muscle reflexly activated differs considerably for different afferent nerves examined on the same test-muscle. Instances given in the list (§ 1) exemplify this. A like result has been noted in a previous paper (2), with, however, use of a different expression for the fractional values.

That in a muscle which is common to them different afferent nerves reflexly activate fractional fields which differ may be of significance for the taxis underlying reflex "local sign." It would be of importance in this connection to determine the relative amount of overlap of the fractional fields of small afferents whose receptive fields adjoin one another or conversely are remote one from the other.

§ (4) For some afferents the muscle-fraction reflexly activated is considerably greater in one of the chosen test-muscles than in the other two. Thus each plantar nerve activates usually >60 per cent. of *tibialis anticus,* but less than 30 per cent. of *semitendinosus,* and certainly less than 20 per cent. of *tensor fasciæ femoris.* Such comparisons were not in our experiments made on the muscles in the same individual preparation, but they accord with other recent observations (2) taken by simultaneous registration of the compared muscles, where the respective contractions were evaluated against each other, though not in terms of their maximal motor tetani. For further instances *v.s.* list in § (1).

§ (5) The fraction of test-muscle activated by a given afferent nerve was found to vary not inconsiderably in the course of one and the same experiment. During the earlier course of the experiment, although the preliminary chemical narcosis had been given full time to pass off, the fraction has often increased, and without explanatory change in general circulation or respiration. Thus :—

Test-muscle.	Afferent nerve.	Hours since spin. trans.	Grm. tension reflex.	Further interval.	Grm. tension reflex.
				Hrs.	
Tensor fasciæ femoris	Peroneal	2	830	3	1300
,, ,, ...	Internal saphenous	2	730	3	1250
Semitendinosus	A dorsal digital	2	433	3	933
,, ...	Another dorsal digital	2	522	3	1110
Tibialis anticus	Internal plantar	2	1475	2	1620
,, ,, ...	External plantar	2	1485	2	1615
,, ,, ...	Internal plantar	2	650	2	930
,, ,, ...	External plantar	2	760	2	900

Such initial depression of spinal reflexes is well-known as a "shock"-effect, in immediate sequence to spinal transection, and also, as regards the flexor muscles, to intercollicular transection (12). The fractions instanced in the list given above have always been the largest observed for the given afferent in the particular experiment. The shock effect disturbs the ratio between individual afferents taken in one and the same experiment less than it is likely to alter that between afferents compared from one experiment to another.

§ (6) The fraction of test-muscle activated by a given afferent nerve, nominally the same from experiment to experiment, has from experiment to experiment varied considerably. Several factors would conduce to such variation. (1) The admittedly frequent anatomical variation from individual to individual in the relation between the spinal roots and the afferent nerves and efferent muscle-nerves. Thus, in one experiment the nerve of sartorius provoked no response in semitendinosus and the internal saphenous scarcely any, nor did strychnine enable a response from the former, thus excluding "shock." Again the amount of reflex response of *tensor fasciæ femoris* to hamstring nerve has varied greatly from experiment to experiment, and in some experiments has been barely perceptible, suggesting segmental limitation. (2) With afferent nerves which are branching freely in the region where stimulated, the finer composition of nominally the same nerve can scarcely be the same for it as stimulated in different experiments. (3) A further factor has been the difficulty, earlier mentioned (p. 449), of determining in some experiments the exact limit of value of the maximal motor tetanus which served as unity for the fractional values in its experiment. (4) Finally there has been the degree of "spinal shock" as a probable variant from experiment to experiment, though care was taken throughout the experimental series to try to regularise this factor by regularity of procedure. The relatively low value obtaining for the reflexes in certain experiments may well have been referable to severer spinal shock or less recovery from spinal shock. In those experiments, however, where a single large afferent has reflexly evoked the maximal contraction power of the muscle as estimated by direct maximal stimulation of its motor nerve there cannot well have existed serious reflex depression. For that reason the fraction-values obtained in experiments giving this guarantee against spinal shock have been chosen for the composition of the list under heading *M. semitendinosus* in § 1 above.

§ (7) That a "central" influence, such as "spinal shock" or "decerebrate shock," can prescribe, for the time being, the size of the reflex elicitable by even maximal stimulation of the afferent nerve indicates that the size limit imposed upon the fractional reflex is referable not to lack of morphologic connections existent between the afferent nerve and its muscle, but to functional restriction of the number of structural connections traversable at the time. And that the fractional numbers which our observations arrive at for the reflexes denote for the most part functional capacity rather than fixed anatomical extent is indicated by several considerations, among them that variability cited in the two preceding sections. In one set of the observed instances, however, the size of the reflex effect in the test-muscle must be regarded as dictated by anatomical limit.

That case is the relatively infrequent one where the afferent nerve has reflexly activated a test-muscle in its entirety, thus reaching the anatomical limit of the effector organ's additive units. Apart from that case, how greatly and extensively the limit set to the fractional size of the reflexes must be a purely functional one, centrally imposed, is emphasised by the influence of strychnine upon the reflexes' fractional size. Strychnine even in subconvulsive dose renders a much larger fraction of the muscle than heretofore reactive to an afferent. This holds both for cutaneous afferents and for proprioceptive. The afferent nerve evokes a larger reflex response than heretofore not merely to stimulations which are submaximal, but also to those which are maximal. Afferent nerves, yielding under maximal stimulation in the unstrychnised preparation reflexes of quite low fractional grade, yield under strychnine, even in subconvulsive dose, reflexes of much greater fractional value, though in our observations still for the most part fractional.

Thus :—

Muscle.	Afferent Nerve.	Reflex in Grm. Tension.		
		Before Strychnine.	After Strychnine.	
Semitendinosus	Internal saphenous	120	600	same experiment
,,	Peroneal	1500	2130	
Tibialis anticus	Musculo-cutaneous	1600	2430	same experiment
,, ,,	Hamstring	540	1960	
,, ,,	Internal plantar	1615	2730	
,, ,,	External plantar	1620	2790	
,, ,,	N. of external sural tric.	370	1020	
		Reflex in Percentage of max. mot. tet.		
		Per cent.	Per cent.	
Semintendinosus	Internal plantar	25	90	same experiment.
,,	External plantar	22	83	
,,	Internal saphenous	26	72	
,,	N. of sartorius	15	80	same experiment.
,,	Internal saphenous	25	75	
,,	Peroneal	73	96	
,,	Peroneal wk. stim. [20 cm. coil]	6 [wk. stim. 20 cm. coil]	40	
,,	Peroneal	92	100	
Tibialis anticus	Internal plantar	52	81	
,, ,,	External cutaneous	37	46	same experiment.
,, ,,	Hamstring	63	77	
,, ,,	Small sciatic	46	63	
,, ,,	Popliteal	80	92	

In our routine experiments, therefore, the size observed for the fractional response of the muscle to maximal afferent-nerve stimulation indicates a limit determined by the degree of conductivity obtaining in the centre at the time. We may ask how far that fractional size, thus observed a few hours after spinal transection, represents a quantitative norm for spinal reactions. In regard to this, the spinal reflexes here measured are ipsilateral and flexor, and "spinal shock" depresses these much less than it does various reflexes of other type (7, 10, 3A). The argument given above in § 6 indicates that in some of the experiments at least there cannot have existed serious depression of the observed reflexes, either from spinal shock or any other cause. We infer, therefore, from the fractional character found for the great majority of our observed reflexes that under normal spinal action the muscular responses are in their respective muscles likewise for the most part fractional, although their fractional values may not strictly correspond with those here stated. Not without bearing on this question seems our experience that production of a full maximal tetanus of the whole muscle not very rarely ruptures the muscle tendon. Under natural use, reflex and other, of the muscle it may be exceptional and unusual for the muscle to be tetanised in its entirety.

§ (8) The values of the reflex contractions obtained severally, by maximal stimuli, from the individual branches derived from an afferent nerve yield a sum which exceeds the value of the contraction evoked, by maximal stimulation, of the whole parent nerve. Thus, with semitendinosus muscle, three of the small dorsal digital branches taken close to their digital clefts yielded respectively the contraction values 2400 gm., 2550 gm. and 1240 gm.; while their parent stem, the musculo-cutaneous nerve containing them and other branches as well, yielded under similar maximal stimulation 2,970 gm. only.

Again: Popliteal nerve-trunk and its branches: test-muscle *tibialis anticus*:

Branches.	Gm. tension of reflex.		Trunk, gm. tension of reflex.
External plantar	1690	7815	Parent trunk, 1820
Internal plantar	1800		
N. of post tibialis muscles	1525		
N. of external sural triceps	800		
N. of internal sural triceps	850		
Branch to small sciatic	1150		

Further, this aggregate given by the popliteal branch-nerves exceeded by more than threefold the gm. tension of the "maximal motor tetanus" obtained from the muscle, *i.e.*, 2350 gm.

There seems little or no evidence that individual afferent fibres subdivide in their course along their nerve-trunks or in such a way that tributary branches of a larger nerve-trunk contain branches of the same afferent nerve-fibre; and such branching would have to be plentiful to account for the above result. Hence that result indicates that the tributary branches of an afferent nerve reflexly activate muscular units possessed by them in common. In other words the fibres of an afferent nerve exhibit the "convergence"-principle (11) in their functional impingement upon the collection of final common paths constituting the motor nerve of the muscle. In the field formed by the muscle's aggregate of fibres the fractions of that field which belong respectively to individual branches of single parent afferent nerve must therefore overlap, in the sense that some of the muscle-fibres must be common to several of the fractional fields; and the amount of this overlap is clearly considerable.

§ (9) The contraction values of the reflexes yielded severally by individual afferent nerves, maximally stimulated and capable of activating the test-muscle, give in sum a value largely in excess of that of the muscle's maximal contraction. This we find with each of the three test-muscles. The amount of the surplus in the case of two of them may be instanced as follows:—

(1) *Tibialis anticus*—maximal motor tetanus; 2460 gm.

Afferent nerve.	Reflex contraction. Gm.
Dorsal digitals, 4 at 900 gm. each (sampled from one) ..	3600
External plantar	1240
Internal plantar	1330
Nerve of quad.-extensor and sartorius	1190
Nerve of sural triceps	300
Hamstring nerve	515
Small sciatic	680
Obturator	565
	10220

(2) *Semitendinosus*—maximum motor tetanus; 3000 gm.

Afferent nerve.	Reflex contraction. Gm.
A dorsal digital	2400
Another dorsal digital	2550
Another dorsal digital	1240
Obturator superficial	1270
Obturator, deep	630
Internal saphenous	1900
Nerve of sartorius	1500
Nerve of quadric, external	2800
External cutaneous (groin)	830
Small sciatic	1860
Anterior tibial	2900
Popliteal	2650
	21130

Had the series of afferent nerves taken been a more numerous one and made up from individually smaller branch-nerves the surplus arrived at would have been certainly larger still. In neither of the above series was the afferent nerve of the test-muscle itself explicitly included, although that afferent possibly contributed, owing to stimulation by the contraction itself. The above results suffice, however, to show that the total aggregate of motor units belonging to the test-muscle is represented several times over in the total collection of the limb afferents which can reflexly activate it. If the values taken above for the reflexes of the individual afferent nerves be replaced by those obtainable under strychnine, their joint excess over the maximal tetanus contraction becomes much greater; in other words the overlap (*v.s.*) of the individual convergent afferents upon the units of their field of effect is seen to be potentially even more extensive yet.

Conclusions.

Our observations, following Camis (1), but by a method other than his, support his conclusions already quoted above, namely, that "the cells of a spinal motor centre" though by reflex action "divided into several independent groups" exhibit for those groups "an independence, which, however, is not absolute." Our experiments extend this result to afferent nerves and test-muscles additional to those examined by Camis. Phrasing the conclusions somewhat afresh, we would summarise thus :—

Each afferent nerve reflexly activating a given muscle acts on a fractional group of the total aggregate of motor-units belonging to that muscle, a fractional group which in relation to the rest of the aggregate may be termed the fractional group proper to that afferent nerve. The units of that group are, however, less or more numerously common to the proper groups of other afferents, the whole aggregate of motor units belonging to the muscle being made up of such fractional " proper groups " commingled. The reflexly available size of each " fractional proper group " fluctuates with fluctuation of the functional state of the spinal centre. Its shrinkage under decerebrate and under acute spinal shock is symptomatic of those conditions, and indicates the dependence of its available size upon influences from other spinal and prespinal centres. The available size of each fractional group is increased by strychnisation ; hence under strychnine the commingling of the " proper " groups becomes more extensive. In certain rare instances the " proper group " of a single large afferent nerve-trunk embraces, even without strychnine, the total aggregate of the muscle's motor units.

Summary.

The reflex contractions obtained in certain typical flexor muscles of the hind-limb by stimulation of various afferent nerves of that limb within a few hours after spinal transection have been quantitatively examined, and show that under that condition the great majority of the individual nerves even when their stimulation is maximal activate the muscle only partially.

The fractional size of the reflex obtainable from a given afferent nerve shrinks under " spinal shock " and under " decerebrate shock," and, on the other hand, becomes much enlarged under strychnisation even of subconvulsive degree.

The tributary afferent nerves of a nerve-trunk yield relatively larger reflexes than does the parent trunk itself.

Inferences drawn are that in its reflex use of a muscle the afferent nerve fractionates it, that the fractionation by different afferent nerves is different, and that the fractions of the muscle proper to the various individual afferent nerves overlap each other in the muscle.

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OBITUARY NOTICES

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Chas W. Andrews.

CHARLES WILLIAM ANDREWS—1866-1924.

Charles William Andrews was born at Hampstead in 1866, and died in London on May 25, 1924, having spent his active official life in the service of the British Museum. He was a graduate in both arts and science of the University of London, and began his career as a schoolmaster. His main interest, however, was in biological and geological research, and in 1892 he was fortunate in being the successful candidate in a competitive examination for an assistantship in the Department of Geology in the British Museum (Natural History). Here he soon began to add original investigation to his curatorial duties, and he eventually became one of the foremost exponents of vertebrate palæontology.

Dr. Andrews at first paid much attention to the fossil birds, of which a useful general catalogue had just been published by Lydekker. He made himself well acquainted with the osteology of birds, and so was adequately equipped for dealing with the large collections of bones of extinct birds which were then being discovered in the surface deposits of lands in the southern hemisphere. In his earliest paper, published in the *Geological Magazine* in 1894, he described some limb-bones of the largest known running bird from Madagascar, which he named *Aepyornis titan*. In subsequent years he made several important contributions to our knowledge of both the Aepyornithes and the fossil carinate birds of Madagascar. At the same time he studied the extinct birds of New Zealand, and a large collection of fossil bird-bones from the Chatham Islands which Lord Rothschild had obtained for the Tring Museum. He pointed out especially that the occurrence of closely related flightless rails in Mauritius, the Chatham Islands, and New Zealand, did not necessitate a former connection between those widely separated lands. The rails might have become flightless independently in the different restricted habitats, and an almost flightless rail, *Nesolimnas*, among the fossils from the Chatham Islands seemed to show that in this form the wings were actually being reduced on the spot. Dr. Andrews also published important new observations on the remains of the Stereornithes and other remarkable extinct birds, discovered by Ameghino in Patagonia, which were received by the British Museum in 1896. To the end he retained an interest in all fossil remains of birds, and his descriptions of an ancestral tropic-bird, *Prophaethon*, from the Eocene London Clay of Sheppey, and a sternum of the largest known flying bird from an Eocene formation in Nigeria, are especially noteworthy.

Soon after Dr. Andrews entered the British Museum, the late Mr. Alfred N. Leeds gradually enriched the collection of fossil reptiles by his discoveries of ichthyosaurs, plesiosaurs, marine crocodiles, and dinosaurs in the Oxford Clay of Peterborough. Opportunity was thus afforded for a more exhaustive study of the Jurassic sea-reptiles than had previously been made. Dr. Andrews turned at intervals to an examination of these fossils, and soon realised that they not only added much to our knowledge of the osteology of the various groups, but also revealed the growth-stages of some of the most characteristic parts. He thus began publishing his observations on them by describing the development of the shoulder-girdle in a plesiosaur (1895). Eventually he prepared a descriptive catalogue of all the marine reptiles in the Leeds Collection, which was published by the Trustees of the British Museum in two well-illustrated volumes in 1910–13. This work forms a solid basis for subsequent research on the subject, and Dr. Andrews himself amplified the results at which he had arrived by later descriptions of new features in the plesiosaurian skeleton revealed by discoveries in the Lias of Northampton and the Wealden of Sussex. His last paper, indeed, read to the Zoological Society just before his death and published posthumously, described and discussed the skin and other soft structures of an ichthyosaurian paddle preserved in a unique fossil from the Lias of Leicestershire.

In 1897 Dr. Andrews' official work was varied for a time by a visit to Christmas Island in the Indian Ocean, to study its geology and collect natural history specimens before the island was despoiled by the phosphate diggers who were about to exploit its resources. The late Sir John Murray had proposed to the Trustees of the British Museum that they should explore the island at his expense, while it still remained untouched, and Dr. Andrews was selected for the task. He spent ten months in the island, and the results of the exploration were published by the Trustees of the British Museum in a small volume in 1900. It proved to be a typical oceanic island, composed of volcanic rocks and coral reefs, and Dr. Andrews' collection included many new forms of animal life which were described by specialists. In 1908 he visited Christmas Island again, also at the invitation and expense of Sir John Murray, to study the changes in the fauna and flora which had taken place during man's occupation, but he found little to report.

After his first visit to Christmas Island, Dr. Andrews began to show symptoms of a disease which handicapped him for the rest of his life, and towards the end of 1900 it was deemed advisable that he should winter in a warm clime. The generosity of one of the Trustees of the British Museum enabled him to spend some months in Egypt. There he joined Mr. H. J. L. Beadnell, of the Geological Survey of Egypt, who was then engaged in examining the Tertiary fresh-water deposits of the Fayûm. Dr. Andrews took part in this congenial work, and his trained eyes soon detected numerous fossil remains of mammals which had not previously been noticed. Among these he recognised two successive ancestral elephants, more primitive than any hitherto known, which he described under the names of *Moeritherium* and *Palæomastodon*. He also found evidence of other new ancestral mammals; and Mr. Beadnell's discovery of the large horned mammal *Arsinoitherium* with other remains in 1902 in the same district, led him to return to Egypt more than once, with funds supplied by the late Mr. W. E. de Winton. Dr. Andrews then studied the whole collection made both by himself for the British Museum and by Mr. Beadnell for the Geological Survey of Egypt, and gave a full account of it, with excellent illustrations by Miss G. M. Woodward, in "A Descriptive Catalogue of the Tertiary Vertebrata of the Fayûm, Egypt," published by the Trustees of the British Museum in 1906. This work contains fundamentally new information on the early ancestry of the Proboscidea, Hyracoidea, Sirenia, and Cetacea, and will always remain one of the classics of vertebrate palæontology.

In subsequent years Dr. Andrews continued his researches on fossil mammals from Africa received by the British Museum, and much extended his original results. He described remains of *Dinotherium* discovered by Mr. C. W. Hobley in British East Africa, and remarkable new hyracoids discovered by Dr. Felix Oswald in the same country. He also made known a remarkably primitive zeuglodont whale from an Eocene formation in Southern Nigeria. At the same time he studied other fossil mammals which were needed for comparison, and his examination and re-description of the type skull of *Dinotherium* from Hesse-Darmstadt may be mentioned as specially important.

Throughout his work Dr. Andrews displayed unusual insight into the meaning of the facts he observed, and all his writings are valuable contributions to science. His descriptions furnish exactly the information needed, and his inferences are always both cautious and adequate. He had a keen appreciation of the value of a fossil, and he avoided overburdening literature with descriptions which had no evident scientific bearing. He was also ever ready to share his learning and experience with colleagues who consulted him, and he was equally helpful and encouraging to visitors who brought specimens to the Museum. His frequently recurring periods of illness never damped his enthusiasm or reduced his powers of work, and his unfailing kindliness endeared him to a large circle of devoted colleagues and friends.

Dr. Andrews was elected a Fellow of the Royal Society in 1906. He was also an active Fellow of the Geological and Zoological Societies, and was awarded the Lyell Medal of the Geological Society in 1916.

A. S. W.

G. A. J. COLE—1859-1924.

Grenville Arthur James Cole, born in 1859, undoubtedly acquired from his forbears some of the tastes which directed his life's work. His grandfather made a hobby of mineralogy, and his father, J. J. Cole, architect to the London Stock Exchange, was deeply interested in geology, and, being skilled in photography, was one of the earliest to realise its value for recording geological features and for registering the progress of geological change.

Cole was educated at the City of London School, under Abbott, and, when in the sixth form, made opportunity to study geology and mineralogy at the Royal School of Mines. Here Prof. Judd, realizing his aptitude, gave him special training, and in 1880 obtained sanction to appoint him as a Demonstrator in the practical laboratory course that, in conformity with the spirit of Huxley, then Dean, he was developing as the first of its kind established in Great Britain. This association lasted ten years, during which the course was elaborated, and many students, who have since become leading geologists, were turned out. Several features of the teaching were crystallized in Cole's 'Aids in Practical Geology,' first published in 1890, a practical work of great value to students, as is manifested by its frequent republication and its present use.

While Cole was at the School of Mines, Judd was working up the results of his studies on the Tertiary igneous rocks of the Inner Hebrides. The two collaborated in an account of the basic glasses of the region, and Cole pursued the subject of Tachylytes further in 1888. At this time, too, Cole became interested in acid glasses, especially in their devitrified representatives found among the ancient English and Welsh lavas, and in the formation and alteration of their spherulitic and lithophysal structures, a subject which brought him into controversy with Prof. Bonney. In this connexion, he co-operated with Butler in work on the lithophyses of Roche Rosse, and visited the Vosges to make a study of the rhyolites there. This work led him later into Hungary, and bore excellent fruit when he came to deal with the rhyolites and associated Tertiary igneous rocks of Northern Ireland.

Before he left London he made other contributions to our knowledge on the Ordovician or, as he thought, possibly pre-Ordovician volcanic rocks, as in his paper with Mr. Jennings on the igneous rocks of Cader Idris, and his study, at first with Sir Thomas Holland and afterwards by himself, of the difficult rocks of Rhobell Fawr. In both cases a firm foundation was laid for the work which has recently been accomplished in these areas. He also visited and wrote on the rocks of Stanner in Radnorshire, without, however, getting out the age of those rocks, a problem still unsolved. His teaching at South Kensington led him to take much interest in devising simple and easily made but efficient apparatus for laboratory tests, particularly for the determination of minerals by flame tests and other methods.

A visit to Mont Genèvre with Prof. J. W. Gregory resulted in a joint paper on the variolitic rocks found there, which led him to look for and find similar rocks and structures in Anglesey and afterwards on the coast of County Down, at Annalong and at Dunmore Head. Cole's last exclusively British paper dealt with the riebeckite-bearing rock of Mynydd Mawr, and the distribution of this mineral, so far as then known, in the country. This included the remarkable discovery that the boulders of riebeckite-rock at Moel Tryfaen, within a mile or two of Mynydd Mawr, had not been derived from the latter massif, but consisted of a different rock-type exactly like that of Ailsa Craig, a type now known to have been widely dispersed as boulders by the "Irish Sea Glacier" of the Great Ice Age. A special feature of his scientific writing throughout his life was his full and generous acknowledgment of the relation of his own work to that of his predecessors in the same fields.

In 1890 Cole was appointed Professor of Geology, later of Mineralogy, and later still Dean of the College, at the Royal College of Science in Dublin, and at once showed himself in sympathy with his new country, not only on its scientific but on its national side. At first, although he was an excellent teacher and a lucid and interesting lecturer, he had not many students, and his school took time to grow. This gave him opportunity to acquire a thorough knowledge of the geology of the country and to study many of the interesting problems of petrology, mineralogy, and physiography which it presents. Thus, he wrote on the influence of geology upon the history and development of the landscape of the Dublin, Wicklow, and Antrim areas, and afterwards took a leading part in excursions of the Geologists' Association to these districts. He also gave attention to the Irish marbles and ornamental stones, as well as to the building-stones chiefly employed in the country, and developed teaching on the application of geology to soils and agriculture. He shared with Prof. Sollas the description of certain pyroxene-bearing calcareous sediments brought by Prof. Haddon from Torres Strait, and saw the possibility that they might throw light on some of the crystalline marbles in which similar minerals occur.

But at this time he was chiefly interested in the origin of obscure and unexplained rock structures, the textures arising from serpentinisation, the sphere-bearing granite of Mullaghderg, cone-in-cone structure, the formation of hullite, and the derivation, alteration, and ultimate destiny of xenolithic rocks and minerals derived by one igneous rock from another. It was this last work which led him to devote several years to the large granitic and gneissose masses of northern and western Ireland. He came to the conclusion that in these cases plutonic magma had invaded and incorporated on a very large scale pre-existing rocks, both igneous and sedimentary. His work did much to harmonise that of Canadian and American workers upon Laurentian rocks with that of the French petrologists on *lit-par-lit* injection and metamorphism.

But Cole's sympathies and interests extended to all branches of geology. He wrote largely on geographical and physiographical subjects, such as the history of the Liffey Valley, the general geography of Ireland, and the 'Narrow Seas and the Arctic Route to Muscovy.' Even palæontology was not neglected for he dealt with Carboniferous and Cambrian (?) forms, and made contributions to the study of the causes of the preservation of shells in one or other of the allotriomorphic forms of carbonate of lime. He was interested in the application of present day operations in elucidating the past, as in the heavy destruction of chalk due to storms in the north of the country, and in the possible causes of the rhythmic deposition of flint in Chalk. His trip to Spitzbergen gave him a new insight into glacial deposition, which was promptly applied to the case of the Irish Pleistocene deposits. He also paid attention to collections of rocks dredged off the Irish Coasts, concluding that there probably existed Cretaceous and Tertiary outliers submerged off the south-west of the country. On this subject a memoir was written after he became head of the Geological Survey.

When in 1905 the Geological Survey of Ireland was placed under the control of an Irish Department, a well-earned tribute was paid to Cole's position as the leading geologist in the country by his appointment as Director, a post he held concurrently with his Professorship to the close of his life. Under his direction special stress was laid on the mineral resources of the country, and on the relations between rocks, soils, and agriculture. Naturally special attention was paid to the bauxites and iron-ores of the Antrim plateau, a study in which Cole's intimate knowledge of the rhyolites and basalts was of vital importance. Maps and memoirs were issued, including a large map of the general mineral resources of the country, and special notices of the Ballycastle, Tyrone, and other coalfields.

Outside, as well as within his official duties, Cole's life was full of activity. He was a prolific and gifted writer, with an easy style, and the knack of conveying information with grace and pleasantry. He was a frequent contributor of reviews or summaries of information to such periodicals as *Nature*, the *Irish Naturalist*, of which he was one of the mainstays, and *Natural Science*. In the last his "Story of *Olenellus*" was a most valuable history of the importance of the discovery of this form in various parts of the world. His descriptions of the geology of Ireland contributed to "Régionale Géologie," now collected into a separate work, have provided the long-needed handbook of the geology of the country.

But besides his text-books, and volumes of a semi-popular character, such as his 'Open-air Studies in Geology,' he wrote many other books with geographical, geological, or social leanings, generally the outcome of cycling or other tours with one or another friend, or with his wife, into the less known corners of Europe, in North Africa, or in Canada and South Africa, which he visited as a member of the British Association. He even wrote some few poems, of no mean merit.

Cole received from the Geological Society the award of the Murchison Fund in 1889, followed by the Murchison Medal in 1909. He was elected a member of the Royal Irish Academy soon after he settled in Ireland, and a Fellow of the Royal Society in 1917. He served as President of Section C of the British Association in 1915, delivering an important address on mountain building, and in 1919 became President of the Geographical Association, while he held the Presidency of the Irish branch of the same association from its foundation till 1922.

Cole's later years were much clouded by illness. He became sadly crippled by arthritis, which, however, he never allowed to interfere in any way with the discharge of his duties or with his habitual hopefulness, his generous helpfulness, and his geniality. Nevertheless life became a very severe struggle, and it was by sheer will-power that he kept alive during his last year or two. These years were also saddened for him by the strained relations between his own and his adopted country, always a source of great grief to him, by the rebellion, and, worst of all, by the closing of his own and other departments in the fine building of the Royal College, the erection of which had been one of the events of his professoriate and a source of legitimate pride. He carried on, however, as best he could at the Geological Survey Offices and elsewhere, and saw to it that, whatever the difficulty and inconvenience to himself, his students suffered as little as the circumstances allowed.

By his death Science and Literature have both suffered a loss which it will not be easy to make good. His friends have to mourn an amiable and lovable personality, full of wit and wisdom, brave and cheery, and withal a man of deep thought and beautiful life.

W. W. W.

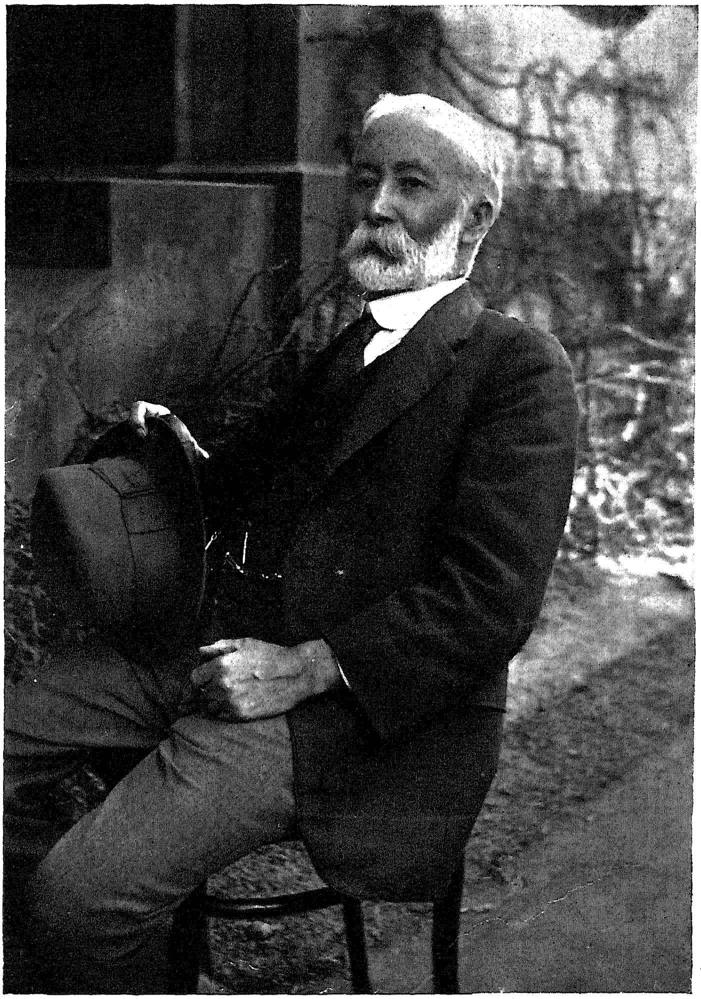
JOSEPH HENRY MAIDEN—1859-1925.

JOSEPH HENRY MAIDEN, I.S.O., F.R.S., F.L.S., was born in London in 1859, received his education at the City of London Middle Class School, and became assistant to the late Prof. F. Barff, from whom he brought a letter of introduction to Prof. A. Liversidge, of the University of Sydney. In this University Maiden became for a short time Lecturer in Botany, after which he was appointed Curator of the newly formed Technological Museum of Sydney. Here he applied himself vigorously to the study of the Australian Native Flora, and personally collected material which formed the nucleus of the Technological Museum Herbarium. He was Curator of this Museum from 1881 to 1896, Superintendent of Technical Education in New South Wales from 1894 to 1896, when he became Director of the Botanic Gardens, Sydney, and Government Botanist and Officer in Charge of the Centennial Park, which positions he retained until his retirement in 1924.

Joining the Linnean Society of New South Wales in 1883, he remained a member up till the date of his death, on 16th November, 1925; was twice President, and thirty-five years a member of the Council. To this Society he contributed no less than 95 papers, either singly or under joint authorship. Among others was a series on the gum and other exudations yielded by various plants. Later, in conjunction with Mr. R. T. Baker, he began a series of papers called "Botanical Notes from the Technological Museum, Sydney," and in 1897, collaborating with the late E. Betche, he started another series, "Notes from the Botanic Gardens, Sydney," describing new species. Through his acquaintance with Dr. W. Woolls, an authority on Australian Eucalypts, Maiden developed a keen interest in this difficult genus, and with Henry Deane contributed a number of papers on the Eucalypts.

Maiden was a member of the Royal Society of New South Wales for forty-two years, having joined in 1883, was Honorary Secretary for twenty-two years, and twice President. To this Society he contributed 45 papers on various subjects, chiefly on the genera *Acacia* and *Eucalyptus*, describing many new species. His last paper, in conjunction with Mr. W. F. Blakely, was read in 1925, and contains descriptions of 16 new species of *Eucalyptus*.

He contributed to the *Agricultural Gazette of New South Wales* many articles on the following subjects:—Forestry and uses of various timbers, ring-barking, fodder plants, poisonous plants, sand-drift problem, particularly as regards sand-binding along the sea beaches, and eradication of weeds. Some of his publications in regard to these matters are "The Weeds of New South Wales," "Useful Native Plants of Australia," "Illustrations of New South Wales Plants," "Manual of the Grasses of New South Wales," and "Wattles and



J. H. Maiden.

Wattle Bark," the last dealing largely with the tannin properties of the genus *Acacia*.

He early saw what an enormous pest prickly-pear would become if not controlled, and on many occasions issued public warnings. From 1911 to 1917 he published fifteen articles on this plant, beautifully illustrated with coloured plates. The investigations of this pest, with a view to its eradication, are being continued by the Commonwealth Institute of Science and Industry.

The Herbarium which he formed at the Botanic Gardens, Sydney, contains a most valuable collection, including specimens obtained by Sir Joseph Banks in 1770, when he visited Botany Bay on Captain James Cook's first voyage. Maiden also took infinite pains to secure for the Sydney Herbarium other type-specimens collected in the early days, the property of European Herbaria. In 1909 he made valuable collections in Western Australia, getting excellent material, the whole of which is not even yet worked up.

His regard for the memory of Sir Joseph Banks was so great that he prepared a biography of Sir Joseph, and had it published for the purpose of raising funds for a memorial to that great botanist.

Maiden was deeply interested in all matters relating to the advancement of science and education ; he was President of the New South Wales Horticultural Society for twenty years, and of the Royal Australian Historical Society for two years. He was a foundation member of the Australian National Research Council in 1919, and a Vice-President at the time of his death.

He was elected a Fellow of the Linnean Society of London in 1888, and in 1915 gained the Linnean Medal, this being the first occasion upon which this medal had been awarded to an Australian. In 1916 he was honoured by having the Imperial Service Order conferred upon him, and was also elected a Fellow of the Royal Society of London, Sir Joseph Hooker, among others, supporting his candidature. In 1921 he was nominated for the Presidency of the Australian Association for the Advancement of Science, but declined it for health reasons, and in 1922 was awarded the Mueller Medal by that body, of which he had been Permanent Honorary Secretary for fourteen years ; and in 1924 the Royal Society of New South Wales conferred upon him the Clarke Memorial Medal.

His "Forest Flora," in 77 parts, is of both economic and scientific value ; but his chief life-work was his "Critical Revision of the Genus Eucalyptus," of which 64 parts appeared before his death, while the remaining parts are now being published. This embodied the results of twenty years of strenuous and patient research. Every species mentioned in this work is accurately and beautifully illustrated. These two publications, and the Herbarium at the Sydney Botanical Gardens, are enduring monuments to Maiden's ability and method. He is also commemorated in one new genus, *Maidenia* (*sp. rubra*), A. B. Rendle (Hydrocharidaceæ), and fifteen new species have also been named after him.

The following appreciation we owe to Dr. A. B. Rendle :—

"It is of interest to note that Maiden approached botany through its application to technology, and we may conclude that he experienced the difficulty which still confronts students of economic botany, namely, that of putting names to the plants with which they are dealing. Maiden set to work to remove this difficulty for his own area, and his most important contribution to botany was his years of work devoted to the collection and careful description of the plants of New South Wales and other parts of Australia. His two outstanding contributions are :—*The Forest Flora of New South Wales*, and the *Critical Revision of the Genus Eucalyptus*. The former, Part I of which was issued in 1902, and Part LXXVII (the last), in 1924, describes and illustrates about 300 trees, or half the number indigenous to New South Wales. The latter (1903–1925) deals similarly with 359 species and hybrids of the most characteristic and difficult of Australian genera. In each case the descriptions are compendia of information, botanical and economic. The Herbarium in the Sydney Botanic Garden, which he built up in the course of his work, is a permanent record of the flora and of his own industry.

"I had met Maiden during his visit to England some years ago, and had also corresponded with him. It was a great pleasure to meet him again in his own domain at Sydney during the British Association Meeting of 1914. Though very busy with executive work in connection with the Meeting, he found time to devote to his fellow-botanists, who remember visits to the beautiful Botanic Gardens on the shores of Sydney Harbour, and excursions to parts of special botanical interest under his guidance. A drive with Maiden through different types of vegetation, as on the trip to Bulli Pass, was a liberal, though strenuous, education. One was impressed by his intimate knowledge of the flora ; he was an observer, not a mere collector.

"Maiden also appreciated the historical side of his science : witness his interest in the work of the pioneers of Australian botany, and his efforts to obtain examples of species from the classic locality. A visit under his guidance to the spot on Botany Bay where Banks and Solander landed from the 'Endeavour' in 1770, was in the nature of a pilgrimage.

"Among the many activities and interests incidental to the busy life of an official botanist in a comparatively new country, it is surprising that Maiden found time for so much genuine spade-work in botany, the work of a pioneer in territory largely unexplored."

As regards Maiden's personality, his many friends testify to his high sense of duty, his loyalty as a friend, his humour and hospitality. His output of work was truly prodigious, resulting from his intense enthusiasm and his exceptionally methodical habits, as shown, for example, in his system of keeping notes, specimens, pamphlets, etc., arranged in excellent order, clearly labelled, in cardboard boxes. In spite of much bodily infirmity of late years, due to rheumatoid arthritis, his mental capacity seemed unimpaired, and with the devoted help of his wife and daughters he worked hard and heroically up to the end.

Those who can speak with authority on his botanical work have the highest opinion of its range and accuracy, and unhesitatingly place Maiden beside those illustrious pioneers in Australian Botany—Joseph Banks, Robert Brown, J. D. Hooker, Allan Cunningham, George Bentham, and F. von Mueller.

R. H. C.
T. W. E.-D.

B. N. PEACH—1842–1926.

Benjamin Neeve Peach was born in 1842 at Gorran Haven, Cornwall, his father being stationed for a time in that county in the Coastguard service. His ancestors came from Northamptonshire. Inheriting from his father, who was a keen naturalist, a great love of natural history, he sometimes joined in hunting for fossils in the Devonian rocks near Fowey. On his father's promotion to Peterhead in 1849, young Peach came to know the contents of nearly every rock-pool along that shore for miles. In 1853 the family were transferred to Wick, where he took a keen interest in the material collected by his father for studying the marine zoology of Caithness and also in hunting for fossil fishes in the Caithness flagstones.

When stationed at Wick, his father discovered a suite of fossils of American facies in the Durness limestones in the north of Sutherland, which immediately attracted attention. Sir Roderick Murchison, then Director-General of the Geological Survey, at once recognised the importance of this discovery, and, as a mark of his appreciation, he completed the education of young Peach at the Royal School of Mines, London. The scientific training at that institution profoundly influenced him, in particular the teaching of Huxley and Ramsay, which gave him a decided bent towards geology. He passed through his classes with such distinction that on Murchison's recommendation he joined the staff of the Geological Survey.

His first official duty was to assist in the determination of fossils in the London office, under the palæontologist, Salter, whose precise methods and wide range of knowledge left a lasting impression on the young geologist. He was transferred to Scotland in 1862, and was associated with James Geikie and John Young (afterwards Professor of Natural History in Glasgow University) in mapping the glacial deposits of the Lothians. At that early date he noted the occurrence of materials from the Highlands in the drifts of the Lammermuirs—a fact which impressed his director, Ramsay, as to the movement of the Highland ice. Peach was convinced that the phenomena of the boulder-clay could only be satisfactorily explained by the action of land ice. Thereafter, with the other members of the staff he mapped the solid geology as well as the drifts. It fell to his lot to survey several of the Scottish coalfields, large areas of the Old Red Sandstone with the associated volcanic rocks, and the eastern portion of the Southern Uplands. In 1879 he was appointed Acting-Palæontologist in Scotland, in addition to his work in the field, with permanent headquarters in Edinburgh. It was then that he assigned to Dr. Kidston the plants, and to Dr. Traquair the fish remains collected by the Survey from the Old Red Sandstone and Carboniferous systems, while he reserved for himself most of the other organisms for determination.

Much as he loved his palæontological work, his great original power was shown in interpreting evidence in the field. The rapidity with which he grasped the essential points in the geological structure of any region and his mastery of minute detail were remarkable. In virtue of these gifts he became the dominating force on the Scottish staff. Sir Archibald Geikie selected him as his companion when investigating special geological problems in the field. These excursions led Sir A. Geikie to place implicit confidence in Peach's interpretation of field evidence.

In 1883 Peach was placed in charge of the survey of the North-West Highlands, and the whole work was carried out under his supervision. He recognised the extreme importance of the researches of Nicol, Lapworth and others, and inspired his colleagues to work with great enthusiasm when unravelling the complicated stratigraphy of that region. He clearly realised that the structures were new to Britain, and that the thrusting of the rock masses had occurred there on a stupendous scale. He saw that the detailed mapping would result in establishing certain fundamental principles in the architecture of one of the oldest mountain chains of Europe. The great controversy between Murchison and Nicol was solved. Nicol's contention was proved to be right, that there is no conformable passage from the fossiliferous Cambrian limestones into the Eastern Schists in the North-west Highlands. The remarkable assemblage of fossils in the Cambrian limestones of that region, first discovered by his father, proved a fascinating subject of study. In his presidential address to the geological section at the Dundee Meeting of the British Association, he suggested the probable palæographical conditions that prevailed in Cambrian time, between Europe and America, in order to account for the American affinities of the Durness fauna.

In 1888, the re-examination of the Southern Uplands by the Geological Survey began, in the light of the succession established by Prof. Lapworth, based on the graptolites. The traverses across the uplands proved beyond doubt that Lapworth's graptolite sequence rested on an impregnable basis. As Acting-Palæontologist Peach had to determine the large collection of graptolites made by the Survey, and he cordially acknowledged the great advance in Scottish geology accomplished by Lapworth.

On his retirement from the Survey in 1905, he spent several years in the preparation of his great monograph on "The Higher Crustacea of the Carboniferous Rocks of Scotland," published as one of the Memoirs of the Geological Survey. This monograph is illustrated by twelve plates of figures reproduced from his own drawings.

One of the prominent features of his character was his readiness to help younger investigators. From his wide experience he gave valuable advice, and, when asked, examined the sections in the field to test the accuracy of the observations. Younger men admitted that contact with his strong personality proved a great stimulus to research.

In recognition of his contributions to geology, he was awarded by the Geological Society of London, the Wollaston Fund, the Murchison Medal, and the Wollaston Medal, the last being the highest honour in the gift of the Society. In 1892 he received the Murchison Centenary Prize, presented by an anonymous donor, in recognition of his "admirable work in unravelling the complicated geological structure of the Scottish Highlands." In the same year he was elected a Fellow of the Royal Society. From the Royal Society of Edinburgh he obtained the Neill Medal. The University of Edinburgh conferred on him the Honorary LL.D. degree.

He died on January 29, 1926, retaining to within a few weeks of the close his youthful buoyancy of spirit and his passionate devotion to geology.

J. H.

J. G. McKENDRICK—1841-1926.

John Gray McKendrick was born in Aberdeen on August 12, 1841, and died in Glasgow on January 2, 1926.

His life is an extraordinary example of what may be accomplished by inborn talent and determination. From whence he inherited these qualities it is impossible to say. This we do know, that both his parents were of Celtic stock, which may help to explain his strong artistic sense and love of music; but none of his relatives on either side appear to have manifested signs of marked ability, or to have attained distinction. His mother died when he was an infant, and his father, who had not succeeded in business, died at the age of 29. He was their only child, and he was left to the care of a good woman who brought him up to the age of 3 years. As he says in the 'Story of My Life,' written for his children in 1919, "I was in a true sense a foundling, and if it had not been that Mrs. Grey took charge of me I would probably have passed out of the world unknown and unknowing."

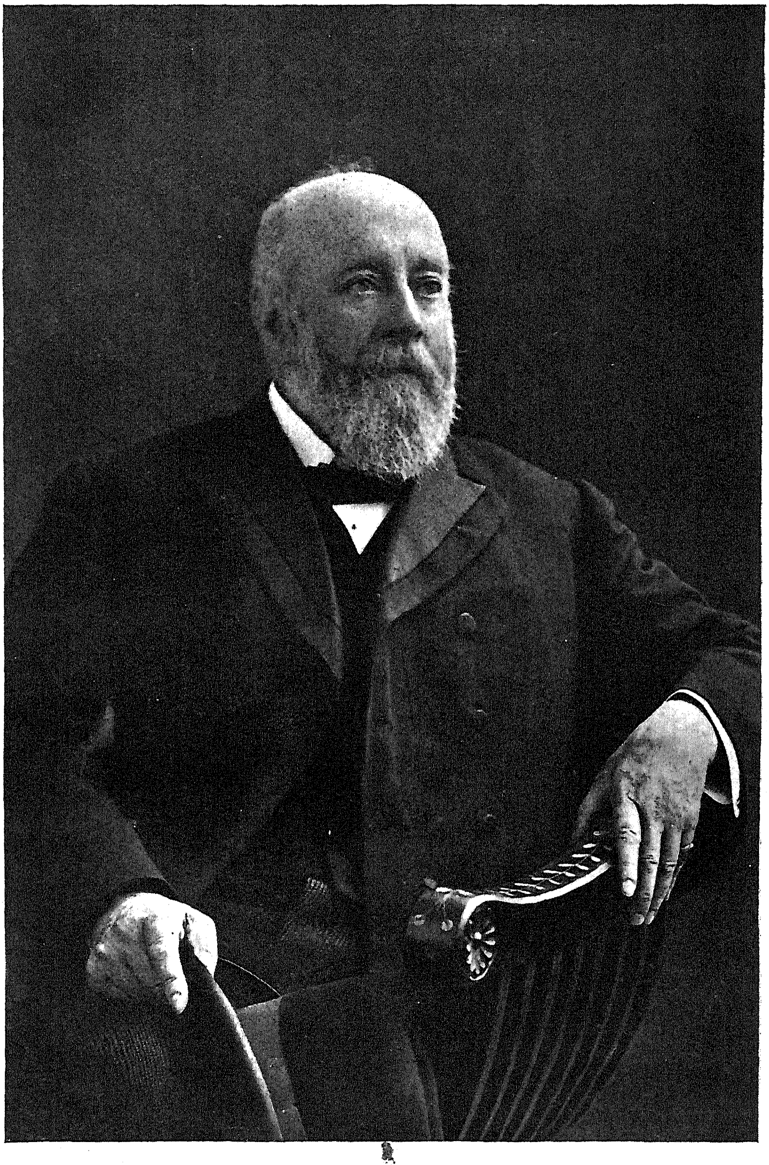
At the age of 3 or 4 he came under the care of his grandparents and an uncle. His early education was obtained at a village school in Perthshire, and at the age of 13 he became a "herd laddie" on the hill near Braco, where nature seems to have made her first appeal to him, for he records his appreciation of the beauty of the landscape and his interest in the nesting habits and cries of the hill birds.

The following winter, through the kindly offices of his early friend Mrs. Grey, he was apprenticed to a small legal firm in Aberdeen, where he served his six years, living on an income which began at £15 and ended at £50 a year.

He must have been an attractive boy, for during these years he became familiar with many cultured families, who introduced him to poetry, music, astronomy, and even to the use of the microscope. In these surroundings he came to realise that his education was very imperfect, and he resolved to remedy the defect. He tells how, outside his seven hours a day at the office, helped by various friends, he began an elaborate course of home study embracing Latin and mathematics. He rose at five, worked till eight, and again in the afternoon and evening.

It was love of knowledge and not academic ambition that urged him on, and his interest extended to Botany and to Zoology, more especially to Marine Zoology. In this he was stimulated by Prof. Redfern, afterwards of Belfast, and he describes the making of an aquarium which he showed at the meeting of the British Association in Aberdeen in 1859, where he first saw Owen, Gould, Huxley, and Murchison.

It was Redfern who suggested that he should become a doctor. He says:



John G. McKendrick.

"The choice of the medical profession arose from the feeling that no education would open the world to me as that placed before one in the medical curriculum." At the age of 20 he passed the Preliminary Examination in the University of Aberdeen, and began to attend such classes as could be taken outside of office hours. In the classes of his first session he gained the First Prize in Comparative Anatomy.

The continuation of medical study and the work of clerk in a law office became increasingly difficult, and the final break occurred through his love of music—a love that grew and developed as his life went on. He went to hear Jenny Lind sing, lost all sense of time, returned late to the office, and was dismissed. Then came what he calls his "Hegira." Aberdeen was no longer financially possible. He rushed to his friend Redfern in Belfast, tried hard to get work which would enable him to study there, failed, and upon the advice of his friend, went straight to Edinburgh, enrolled in medical classes, and after a long dreary hunt found work in the office of a newspaper now long extinct. Here, for 12*s*. a week, eight hours a day were demanded, but they could be put in "when I liked," which enabled the medical classes to be attended. He came under the influence of John Goodsir, and won the medal in Anatomy. This at once opened the prospect of an increased income from private coaching, and fortune seemed to smile upon him. But, alas, a pulmonary hæmorrhage cut short the gleam of hope and banished him from his studies to some months of country life. His health was restored, and he resumed work at Aberdeen, where he acted as Assistant Librarian in the College, and made the acquaintance of such men as David Ferrier and Robertson Smith. But the difficulty of ways and means again drove him to Edinburgh, where more could be made by coaching. He recounts how at this time his interest in acoustics was roused by Alexander Crum Brown, whom he found engaged in Wheatstone's experiment of transmitting sound.

In 1864, he graduated at Aberdeen, and he soon obtained an appointment as Visiting Medical Officer at Chester General Infirmary. From this, he obtained the post of Resident in the Eastern Dispensary, at Whitechapel, at a salary of £100 without board, and with unfurnished rooms. In his spare time he attended lectures on Comparative Anatomy by Huxley, and on Chemistry by Hoffmann. His interest in Zoology is indicated by his application for the Prosectorship at the Zoo. The appointment he finally obtained was that of Resident Surgeon at the Belford Hospital, Fort William, Inverness-shire—a very pleasant change from the slums of Whitechapel.

In 1867, he married Mary Souttar. It was in 1869, that Hughes Bennet, Professor of the Institutes of Medicine (Physiology) in the University of Edinburgh, was called to a consultation at Fort William. McKendrick and he discussed experimental work on calomel, which had been carried out by William Rutherford, Bennet's assistant. Apparently, as a result of this, Bennet later invited him to become one of his assistants at a salary of £100 a year. In spite of being married, and already having a child, he accepted.

His meagre income in Edinburgh he augmented by coaching for the Final Examination, and he says "teaching of this kind I found invaluable to myself. and it prepared me for the duties of a lecturer, as it led to lucidity and also to brevity of expression." His life as a physiologist thus began when he was nearly 30 years of age, an age at which it is not easy to acquire a new mental outlook. So far, he had had no training or experience in the methods of experimental science, and the atmosphere in which he found himself was not conducive to its development. Hughes Bennet was essentially a physician. He had aspired to the Chair of Medicine, and his heart was never in Physiology. He was ignorant of, and despised the use of the apparatus of precision, which Goodsir had been instrumental in introducing from Germany. Apparently, a certain von Pezold had to be imported from Berlin to teach its use to the staff of the Physiology Department.

In spite of these disadvantages McKendrick accomplished a considerable amount of research, which he communicated to the Royal Society of Edinburgh, and which was published in their 'Proceedings.' Unfortunately, the circulation of this publication, in which so much valuable work has appeared, is all too limited, and his investigations hardly received the attention they deserved. His early interest in music led him to investigate the perception of musical sounds in musical and in unmusical people. In 1871, he began an experimental investigation of the functions of the corpora striata and cerebral hemispheres in the pigeon, the results of which were published in 1873.

A friendship formed with James Dewar, then an Assistant in the Chemical Department of the University, led to an investigation on the influence of light upon the eye.

It was probably suggested by the work of du Bois Reymond and others upon the electrical phenomena of muscle and nerve, a development of the older work of Nobili and Matteucci. The results were communicated to the Royal Society of Edinburgh in a series of papers from April to June, 1873. Unfortunately, the authors were not aware of the previous work by Holmgren, published in 1866, but their investigations not only confirmed, but also extended his results, although they left the problem of the cause of the electrical variations observed practically unsolved.

The co-operation between physiologist and chemist was continued and resulted in a series of studies of the physiological action of the chinoline and pyridine bases, which were published in 1875. There is little doubt that this work formed the basis of subsequent investigation in Germany of various therapeutic agents, such as antipyrin.

Bennet's failing health threw most of the teaching upon McKendrick, but when Bennet resigned, in 1873, Rutherford was brought from King's College, London, to succeed him. This threw McKendrick on his beam ends, but he courageously decided to continue teaching physiology. He purchased from Bennet his diagrams, microscopes, etc., started as an extra-mural lecturer, and was appointed Lecturer at the (Dick) Veterinary College, while he also increased his income by lecturing to ladies. All this involved an immense amount of work and left little time for research.

McKendrick seems to have made but one visit to the laboratories of Europe, and he records that he then saw Max Schultze, Pflüger, du Bois Reymond, Helmholtz, Ludwig, and Koenig.

In 1876 he was appointed by the Crown to the Chair of Physiology in Glasgow in succession to Buchanan, who is best known by his work on the coagulation of the blood.

In Glasgow McKendrick found no adequate accommodation, and practically no facilities for teaching. There was nothing except "a few ancient microscopes and a sphygmograph." The value of the chair was at first between £400 and £500 a year, with no provision for expenses or salary of assistants, and for long his application for a grant was not favourably considered by the Senate. But he brought with him the apparatus he had purchased from Hughes Bennet and he faced the situation, started practical classes and acquired microscopes and other apparatus. Soon his classes increased, and in 1893 his income was further augmented and stabilised by the University Commission.

Through the good offices of his friend, Sir James Dewar, he was appointed Fullerian Professor at the Royal Institution in 1881, and lectured there for two years. In addition to his University work he did much popular lecturing, and this left little time for research. In 1877 the British Medical Association appointed a committee to investigate the action of anæsthetics, presided over by McKendrick, and their report was published in the 'British Medical Journal' of December 18, 1880. The development of the phonograph interested him greatly, and from time to time he published the results of various attempts to analyse the records.

Teaching rather than research was his forte, and he, in Glasgow, and Rutherford, in Edinburgh, were among the pioneers of the modern method of practical instruction in physiology. McKendrick was certainly one of the best popular lecturers of his time. He had the gift, to an unusual degree, of holding a popular audience, and the ingenuity and success of his demonstrations always delighted the spectators. He was also an excellent teacher of medical students, and his experience of medical practice had given him an insight into their requirements, so that they always impressed their examiners as thoroughly trained on the right lines for the future study of medicine.

He wrote several books and many articles, and as an author he was always clear and interesting. Probably his best-known work is his 'Life of Helmholtz' (1899). His large 'Text-Book of Physiology,' in two volumes, appeared in 1888 and 1889.

He was elected a Fellow of the Royal Society in 1884, and served on the Council in 1892–93.

His great ambition, to secure for Glasgow University a properly equipped Physiological department, was realised in 1903, when the generosity of the Glasgow public put the necessary funds at the disposal of the University, and he had the satisfaction of seeing the buildings for the present Institute completed before he resigned.

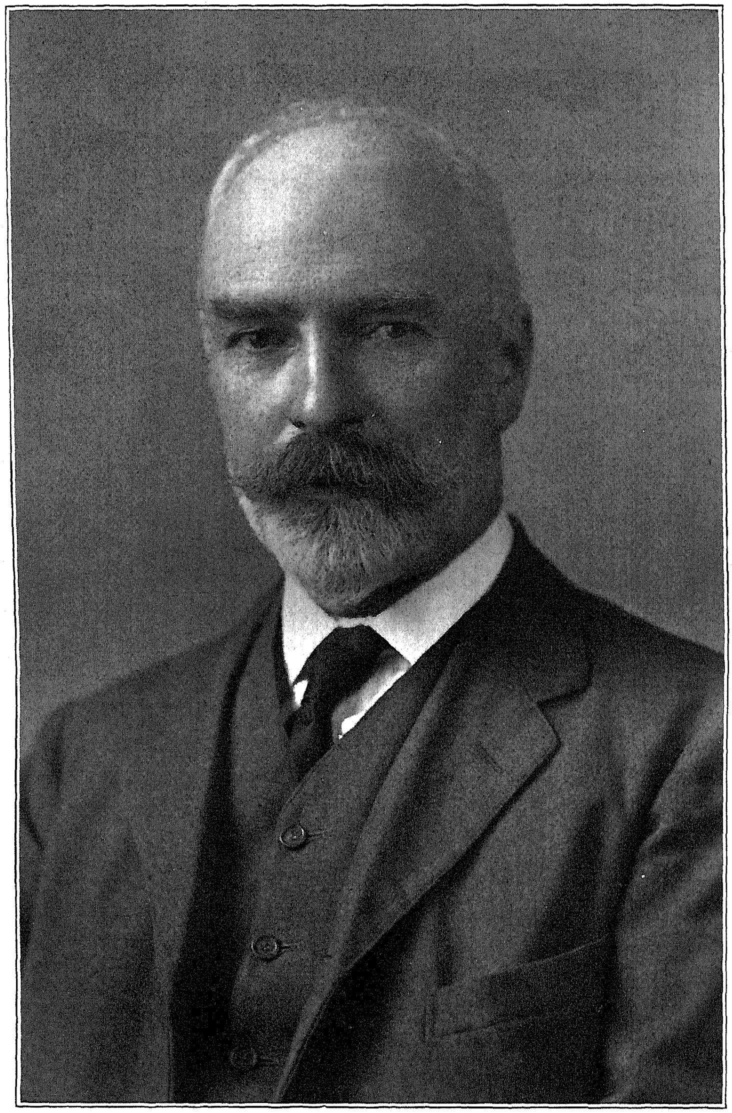
With characteristic unselfishness he recognised that the equipment of the Institute should be left to his successor, and in 1906, after thirty years of service, he retired to his well-earned leisure.

Private sorrows, bravely borne, had fallen upon him as his ambitions for his science in the University were being realised, and it was with a saddened heart that he transferred his home to Maxieburn, at Stonehaven, which he had so long looked forward to as the centre of the family life in the midst of which he was so happy. In his retirement he kept his lively interest in science, and during the war, when younger men were doing their duty, he had the satisfaction, in spite of his age and infirmities, of doing his "bit" by acting as Examiner at Aberdeen University.

His genial, kindly nature and wide culture brought him an extensive circle of friends of all classes. On his retirement they were able to show their admiration and affection by providing funds for a Laboratory of Experimental Psychology, in which the work on the special senses, to which he had made such valuable contributions, might be continued and extended.

McKendrick was without selfish ambition. To him his science and his University were everything, and he has gone to his long rest with his life's work well accomplished.

D. N. P.



Arthur R. Cushny.

ARTHUR ROBERTSON CUSHNY—1866–1926.

A. R. CUSHNY, who died suddenly on February 24th of this year, at the early age of 60 years, was by common consent the leading Pharmacologist of this country. The course of his career, moreover, had taken him into other countries, where he was known and honoured as in his own, so that his reputation and authority in his own subject, and in a wider field of experimental medicine, were truly international.

Cushny was the fourth son of a Scottish clergyman, and was born on March 6th, 1866, at Fochabers, in Morayshire. He passed from the local school to the University of Aberdeen, where he graduated M.A. in 1886. Before this graduation he had already chosen the profession of medicine, and had entered as a student at the Marischal College, from which in 1889 he graduated M.B., C.M., with the highest honours.

At that time there were but few centres in Britain in which Pharmacology was presented as a definite branch of experimental science by a teacher devoting his whole time and interest to the subject. Aberdeen, however, was of the few, and Cushny had the good fortune to come under the influence of John Theodore Cash, to whose teaching he attributed the awakening of his interest in Pharmacology, and his early decision to make it his life's work. Accordingly, when Cushny in the year of his medical graduation was awarded the George Thompson Fellowship by his University, and left Britain, at the age of 23, to study on the Continent, he had already a definite aim; and the choice thus made, under the influence of Cash, was soon to be confirmed by an association lasting several years with Oswald Schmiedeberg. His first object, however, on leaving Aberdeen was to widen his experience of physiological technique. He spent a year in Berne, studying under Kronecker, and a short period in Würzburg, before settling in Strassburg, then the most famous centre of pharmacological teaching and research in Europe. Schmiedeberg, indeed, had done more than any other one man to promote the application, in German-speaking countries, of the newly developed methods of physiology and biochemistry to the study of the action of drugs and remedies, and thus to replace the traditional "materia medica" and empirical "therapeutics" of the old medical curriculum by the experimental science of Pharmacology; and the new tradition, which he did so much to create, was carried to other countries largely by his pupils. With Schmiedeberg, therefore, Cushny was bound to find full opportunity for following his chosen course.

After a year's research work as a guest in the Institute, he accepted appointment as assistant to the Professor, and remained in this position during 1892 and 1893. As co-assistants, whom he always kept in affectionate remembrance, he had C. G. Santesson and E. P. Poulsson, professors of Pharmacology now in Stockholm and Oslo respectively, and Fr. Pfaff, formerly professor of Pharmacology at Harvard. Cushny's own publications direct from the Strassburg Institute included papers on anæsthesia with chloroform and ether, on the active principles of gelsemium, and on the action of muscarine on the frog's heart. He had also begun investigations on sparteine and on the poison of castor-oil seeds, which were finished and published after he had migrated elsewhere. For Schmiedeberg he formed, and always kept, a deep admiration and regard, which found public expression when, in due course, he dedicated his own great text-book "dem Meister vom Schüler." At the same time Cushny probably had, towards the end of his period in Strassburg, a conviction which he certainly held in later years, that the influence of Schmiedeberg and his school, like that of most reformers, had caused the pendulum to swing for the time too far. The new Pharmacology, with its sceptical aloofness from therapeutic effects which could not be analysed by laboratory methods, was tending to lose contact with practical medicine. That some feeling of this kind was growing in Cushny's mind, on the eve of the next important step in his career, is strongly suggested by the fact, recorded by Prof. J. J. Abel, that in 1893 Cushny had planned to return to Britain, and to seek opportunity for combining laboratory research with clinical work in a hospital and consulting practice. Abel himself had just vacated the chair of Pharmacology at the University of Ann Arbor, Michigan, on appointment to that at Johns Hopkins, and had been commissioned, on visiting Europe, to find a successor. Apparently he had some difficulty in persuading Cushny to accept a position which would afford no direct opportunities of contact with clinical work, and would cut him off definitely from medical practice. However that may be, Cushny accepted the invitation to Ann Arbor, and left Strassburg, at the age of 27 years, for the Chair which he was to hold with great and growing distinction for the next twelve years.

In the first few years at Ann Arbor, so far as the published record gives indication, the new professor would appear to have been engaged in completing investigations of somewhat minor importance, already begun at Strassburg. In addition to some already mentioned, there is a paper in 1896, in the first volume of the newly founded *Journal of Experimental Medicine*, on the toxicity of piperidine, coniine and intermediate bases. By this time, however, Cushny must have already begun his attack on one of the major problems of Pharmacology, which was to hold his interest for the remainder of his life, with results which, by themselves, would have given him high rank among workers in Pharmacology and Therapeutics. In the second volume of the *Journal of Experimental Medicine*, in 1897, he published a long and important paper on the action of substances of the digitalis series on the circulation in mammals. The pharmacological literature on the series was already enormous, but, as Cushny pointed out, the direct experimental observations of their action on the heart had almost all been made on cold-blooded animals, the demonstration of their action on the circulation in mammals having been practically confined to records of the arterial blood-pressure. The work of Roy and Adami on the use of the myocardiograph had appeared shortly before Cushny's migration to Ann Arbor, and the new method gave him the opportunity of directly recording the effects of digitalis and its allies on the muscular contractions of the different chambers of the mammalian heart. This paper, giving the results of Cushny's first completely independent investigation, in a field of his own choice, has rightly come to be regarded as a classical contribution to the subject with which it deals. As indicated above, it was the first of a series, published at wide intervals, dealing with one of his chief scientific interests. His mature views on the subject were presented in a monograph on "The Action and Uses in Medicine of Digitalis and its Allies," published as recently as 1925.

Having completed his masterly account of the action of digitalis on the normal mammalian heart and circulation, Cushny was evidently impressed with the lack of clear knowledge concerning the defects of the heart's action, responsible for the various forms of irregular pulse encountered in human patients. In the absence of such knowledge no rational transfer could be made of his pharmacological data to therapeutic practice. Those were days before the polygraph and the string galvanometer; conditions which, through the work of Mackenzie, Lewis and others have now been so thoroughly analysed, seemed then to present an inextricable tangle. Cushny, therefore, set himself the task of producing artificial irregularities in the mammalian heart by direct stimulation of its chambers, and of comparing the irregularities of pulse thus produced with those recorded by the sphygmograph in the clinic. A preliminary paper on some of the physiological results was already published with S. A. Matthews in 1897, but the full exposition of their significance, in the interpretation of pulse records, was not given till 1899 (*Journal of Experimental Medicine,* vol. 4). In this paper Cushny, by comparing the records of irregularities produced experimentally with those shown in pulse records obtained from patients, was able in the latter to distinguish premature contractions of the auricle from those originating in the ventricle. These premature contractions had simultaneously been identified by Wenckebach, who called them "extra-systoles," and similarly identified their points of origin, with the aid of Cushny and Matthews' published physiological records. The successful analysis of these recognisable types of intermittence occupies the main part of Cushny's paper, and shows him to have been well in advance of other workers at that time. It was really transcended in importance, however, by a suggestion which, with characteristic modesty and reserve, he put forward in a short paragraph supplementary to his main thesis. "A few words," he wrote, "may be added in regard to the extreme irregularity of the heart known clinically as delirium cordis . . . . The clinical sphygmogram in these cases resembles exactly that obtained from dogs when the auricle is undergoing fibrillary contractions, which may be continued for a long time without proving fatal. I do not wish to assert that the clinical delirium cordis is identical with the physiological delirium auriculæ, but the resemblance is certainly striking."

It were idle to speculate on the change which might have been produced in the history of cardiology during the ensuing ten years, if Cushny had felt sufficient confidence in its value to follow up this suggestion. Already he held the clue to the meaning of an important type of clinical irregularity, and that type was pre-eminently the one in which benefit was to be obtained by the action of drugs of the digitalis series, which had formed the starting-point of Cushny's whole investigation. Probably the clinical recognition of the condition was not yet sufficiently definite to make Cushny feel sure of his ground; for whatever reason, he did not pursue the investigation at the time. Some seven years later, in conjunction with C. W. Edmunds, he again drew attention to the practical identity of the pulse-record from a particular case of this persistent irregularity with that obtained from a dog, in which auricular fibrillation had been experimentally produced. At this later date clinical cardiology was showing a rapid advance, largely owing to the introduction and application of the polygraph by James Mackenzie. From evidence obtained with that instrument, Mackenzie attributed the condition of persistent irregularity to origin of the rhythm in the auriculo-ventricular node, having apparently considered the possibility of auricular fibrillation, but rejected it, through ignorance of Cushny's evidence, or failure to appreciate its significance.

It was not until 1909 that Lewis in this country, and Rothberger and Winterberg in Vienna, independently established, by the use of Einthoven's string galvanometer, the correctness of the suggestion originally made ten years earlier by Cushny. Mackenzie also was then quickly convinced that the condition which he had called "nodal rhythm" was the result of auricular fibrillation; and, since an important feature of his own work during the preceding years had been to demonstrate that the cases of "nodal rhythm" were those in which digitalis and its allies showed their most definite therapeutic action, this conclusion could now be transferred to the condition of auricular fibrillation, which Cushny had so many years earlier produced experimentally, and tentatively recognised in the clinic. There remained the problem of explaining this therapeutic effect, in terms of what was known of the mode of action of digitalis, and to this Cushny devoted much further investigation. He welcomed the opportunity of collaboration with Sir James Mackenzie in clinical observations, and returned to the question more than once in laboratory research, seeking always to define the parts played in the therapeutic result by the different aspects and phases of the action of digitalis on the normal heart, as he had clearly differentiated them in his earliest paper on the subject.

Cushny's work at Ann Arbor was by no means limited to these fundamental investigations on the pharmacology of digitalis and the therapeutics of heart disease. With G. B. Wallace he published investigations on the action of saline cathartics, he wrote with characteristic balance and sanity on the use of alcohol in therapeutics, and he had already entered on the two other main lines of investigation with which, together with those on digitalis, his name will probably be most clearly and permanently associated—that on the contrasted actions of optical isomers, and that on the mechanism of urinary secretion. Beyond all this, in 1899, the year of his great paper on cardiac irregularities, he completed and brought to publication his "Text-Book of Pharmacology and Therapeutics." The only real predecessor to this book, in the same field, was the "Grundriss der Arzneimittellehre" of Cushny's teacher, Schmiedeberg. Something in its general scheme, and in its approach to the subject from the experimental side, Cushny's book was bound to owe to this model. The debt, however, was only of this general kind, and it may safely be claimed for Cushny's great work that it was the first text-book of the subject in any language to consider and to meet the needs of student and scientific physician alike; for, while taking his stand firmly on the results of exact experiment, Cushny had always the practical end in view, in accordance with the book's sub-title—"The action of drugs in health and disease." The book rapidly established itself with teachers, students and practitioners in the English-speaking world, and has played a very important part in the great development of pharmacological teaching and research since its publication. Throughout the rest of his life Cushny worked steadily to improve his book and to embody in successive editions the latest results of sound investigation. By constant resifting of the older work, and replacement of what was obsolescent, either in the list of remedies or in the evidence concerning their action, by what was of fresh value or importance, he contrived that the book should retain its original scope and character through all changes of detail, and in spite of the enormous growth of the published evidence calling for consideration.

In 1905 University College, London, decided to found a full professorship in Pharmacology. Teaching in the subject had previously been given by one of the physicians on the staff of the associated hospital; and the arrangement had, indeed, given opportunity to one, at least, who was by instinct and achievement a great experimental pharmacologist, though practising as a physician—Sidney Ringer. The subject, however, was attaining such independent importance that a man was needed who would give his whole time to teaching it, and to promoting research in it by his influence and example. Cushny was induced to accept the new Chair, and held it for the ensuing thirteen years. In this case he did not, as at Ann Arbor, succeed to a department already well organised and housed; he had to begin at the beginning, with no immediate prospect of more than one ill-lit room for his own research. Though accommodation was poor, however, the calls on his time for teaching and administration were relatively few, and he was soon busy again with the relation between optical isomerism and pharmacological action, with a new investigation of the action of drugs on the uterus, and, a little later, with the chemotherapeutic properties of antimony. The events already mentioned brought him back again, in 1909, to the study of cardiac irregularities and digitalis, in conjunction with Mackenzie and other clinical colleagues. In the circle of his more immediate colleagues, in the neighbouring faculties at University College, he soon established himself on a footing of close scientific and personal fellowship. In spite of his quiet and unassertive manner, his influence quickly made itself felt in various directions, and in scientific societies and Government departments alike his opinion was sought on all kinds of matters touching on Pharmacology. Later when, through a generous gift of the late Mr. Carnegie, Cushny was able to build a new and well-equipped Institute alongside that of Starling, younger pharmacologists from other British schools came to him as assistants, and from other countries as guests. He published an important paper on the alkaloids of Senecio (rag-wort), which he identified as the cause of hepatic cirrhosis following fodder-poisoning in cattle, a short one on nutmeg-poisoning, and a series, with different collaborators, on the pharmacology of the respiratory centre.

In the years immediately preceding the war he had returned to his investigation of the mechanism of urinary secretion, and was gathering and sorting the material for the important monograph on that subject which was published, as one of the series edited by Starling, in 1917. As a critical review, clear, cautious and fair, it immediately gained wide appreciation. This monograph and his text-book show Cushny as a writer at his best. He had a keen eye for the heart of a problem, and a truly remarkable faculty for reducing a bewildering mass of conflicting evidence to a coherent, balanced and readable statement. His literary style was exactly suited to his purpose. He used language with economy and scholarly precision, avoiding rhetoric as he shunned any kind of personal display. The most he allowed himself was an occasional glint of quiet humour. A few sentences may be quoted from the letter to Starling, as general editor, printed by way of preface to the monograph. They give a hint of the immense and conscientious labour which Cushny gave to this task, and are so characteristic of his manner and his attitude, that one who knew and loved him can almost hear him speak with his quiet chuckle. "No other organ of the body," he writes, "has suffered so much from poor work as the kidney, and in no other region of physiology does so much base coin pass as legal tender. It was therefore necessary to sift thoroughly this mass of printed matter of over 6,000 pages, and I have read it carefully and, as far as might be, sympathetically, though I must confess that my patience has been sorely tried by some papers in which the depth bore no proportion to the length. . . . And as my criticism had to be brief, it may sometimes appear more severe than I had designed;

anyone who may feel himself aggrieved by it may find some consolation in the pages in which your own earlier papers and my own are critically discussed, where he will find that they also do not pass through the ordeal unscathed."

The theory of kidney secretion which Cushny himself put forward, and modestly termed the "modern" theory, was for him merely a rational framework, enabling him to make an orderly presentation of the existing knowledge. Like Starling, he had started with the view that an application of newer physical knowledge would suffice to rehabilitate Ludwig's theory, of filtration in the glomeruli and reabsorption in the tubules, in place of the Bowman-Heidenhain theory of vital secretion. His examination of the possibility produced evidence which any later speculations must take into account; in particular, Cushny's distinction of "threshold" and "non-threshold" substances will remain as a solid contribution to the subject. In the end he found himself driven, by the evidence of his own observations, to credit the tubule cells with a selective action. Part of the reabsorption theory seems to be more firmly established as evidence accumulates, but the question as to whether the non-threshold substances are concentrated wholly by a selective failure of reabsorption, as Cushny held, or are to any extent newly secreted through the tubule cells, remains for final settlement. Cushny's monograph brought the evidence to a new focus, and for many years will continue to provide a basis for further investigation.

Another monograph, dealing with the third chief interest of his working life, has yet to appear. This embodies the Dohme lectures given in Baltimore in 1925 on "The Biological Relations of Optically Isomeric Substances." In a series of papers, of fundamental importance for any ultimate theory of the nature of pharmacological action, Cushny had shown that the tissues of the higher animals, in their response to the intense and precisely localised actions of certain alkaloids and bases—the hyoscyamines, the hyoscines, the adrenalines—exhibit a selective relation to optical isomers, recalling that which Pasteur had long ago demonstrated for the unicellular organisms. The forthcoming monograph will give new opportunity for appreciating the significance of the whole series of related phenomena, in the calm light of Cushny's critical wisdom.

The war meant for Cushny, as for most others, a diversion of activity into new and unexpected channels. He worked hard on committees, of the Royal Society among other organisations, and responded loyally to the call soon made on Pharmacology, hitherto among the least warlike of the sciences. Undoubtedly he felt the strain which bore upon all, and the breaking of sympathies and personal contacts which he had prized.

In 1918 Cushny was invited to the chair of Pharmacology in Edinburgh, in succession to Sir Thomas Fraser. Fraser had held a joint chair of Materia Medica and Clinical Medicine, and it was proposed that the new Professor, while devoting himself to Pharmacology, should work in close association with a Professor of Clinical Therapeutics, who would give his whole time to investigation in the hospital. The post was therefore one which offered, not only a wider teaching opportunity than that at University College, but the promise of that regular contact with practical therapeutics which Cushny had always desired. Something of a Scotsman's hunger for the air of his native land probably helped to weigh the balance, and he decided once more to migrate. He adapted himself with his usual quiet efficiency to the new conditions, and was soon a trusted and influential adviser in the affairs of the Medical School and the University. During those last eight years there was no definite relaxation of his quiet, steady work in the subject of his choice. His two other monographs were written, in addition to new editions of his text-book and a series of original papers. He served on two international conferences on the biological standardisation of drugs—a method which he, indeed, had initiated during his period at Ann Arbor. He took a prominent part in the arrangements for the International Physiological Congress in Edinburgh in 1923, and showed himself eager, with gracious hospitality, to promote the renewal of scientific friendships which the war had interrupted. In 1925 he again travelled to the United States, to give the Dohme lectures at Baltimore, to receive an honorary degree from his old University, and to revisit many old friends. Only a few who knew him closely would have noticed a gradual withdrawal from the risks of fatigue and excitement in one who had always shunned the appearance of exertion. These few knew that for some years, through the chance of a class-room observation of his blood-pressure, he had been aware of a danger threatening his life. When the end came it was sudden, as he would have wished, and free from the lingering disability which had been his only fear.

Nobody can make a careful study even of Cushny's published work without obtaining some impression of his personality. Such a student will realise him not only as a man of deep and exact knowledge, and of ripe experience, but as one in whom pride and modesty combined to make it impossible that he should press the claims to recognition of his own discoveries. Probably few among the many who had a general sense of his authority and eminence in his own subject have appreciated the full importance and originality of his contribution to the therapeutics of heart disease; still fewer, probably, know that Cushny first predicted the importance of antimony as a specific agent in protozoal infections, and himself produced the first suggestive evidence of its value, leaving to others, who rapidly invaded the territory thus opened, the detailed exploration, with all that it has meant for the control of tropical diseases. He had, indeed, an almost exaggerated fear of anything like display or pretention; one had to penetrate the veil of a natural reserve to appreciate fully the solid worth of his character. He did not give his intimacy easily, but responded with a true warmth of affection when his confidence was won. The generosity of his help, to younger workers in his own field, none knows better than the writer of this notice. At a time when he hardly knew me, on my publication of a paper dealing with work in a field which he had himself begun to explore, he sought me out, with spontaneous warmth of approval and encouragement. As I came to know him better, it became natural to me to submit my observations or ideas to the test of his opinion. As many others must have done, I always found him generous with his interest and his time ; cautious and critical. he kept his mind open to new ideas, and seasoned his comment with a kindly humour. In the presence of loquacity or exaggerated enthusiasm, however, he retired behind a screen of whimsical boredom. To know him was to trust him absolutely, and to understand what it was which gave him such weight and authority with his students, and with colleages at the council board.

Cushny received honorary degrees from his old University of Aberdeen, and from that of his first professorship at Ann Arbor. He was elected to the Fellowship of the Royal Society in 1906, and served on its Council from 1916–1918.

Early in his period at Ann Arbor, Cushny married Sarah Fairbank, a young English lady whom he had met in Strassburg. Their home life was one of serene happiness and of charming hospitality to his students and colleagues. During his tenure of the Chair at University College they lived, with their one daughter, in Hampstead ; later, on removal to Edinburgh, they were able to secure an historic manor house, not far from the city, with a large and beautiful garden —Peffermill House, at Craigmillar, the "Dumbiedykes" of the Heart of Midlothian. Cushny's pleasure in this house, and especially in his garden, grew with the years, and he had begun to look forward to a quiet time in the future, when he could withdraw from public activity, and commune with the fathers of therapeutic science, in his library and among his plants ; but the blow fell suddenly, and he passed from the fullness of his powers into the final sleep.

H. H. D.

SIR FREDERICK MOTT, F.R.S.—1855–1926.

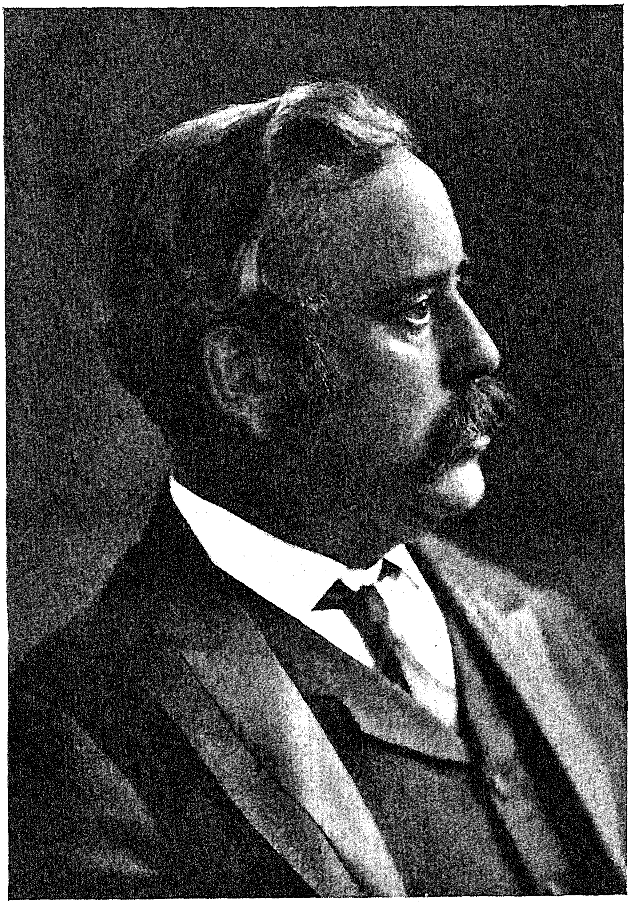
FREDERICK WALTER MOTT died on June 9, 1926, in his seventy-third year. He studied medicine at University College, London, where he had as contemporaries W. D. Halliburton, Victor Horsley, Leonard Hill, Sidney Martin and others who, like himself, afterwards attained eminence in science and medicine. Both there and at the University of London, where he graduated M.D. in 1886, he obtained many distinctions. He was elected to the Fellowship of the Royal Society in 1896, and served on the Council in 1908–1909.

Mott laid the foundation for his subsequent study of the diseases of the nervous system by systematic researches into its normal structure and functions, and obtained a notable position in physiology before he devoted himself to pathology. The effect of these studies upon his later work is manifest.

Most of his earlier work was carried out in the Physiological Laboratory of University College, and subsequently at King's College, much of it in conjunction with others. These early investigations included the effect of stimulating the cerebral cortex of the monkey on the movements of the eyes—with the present writer—the question whether bacteria occur in healthy tissues—with Victor Horsley—the effect of acute anæmia on the brain—with Leonard Hill—and the chemical substances set free during nerve degeneration, and their physiological effects—with Halliburton. Somewhat later he investigated, in conjunction with Sherrington. cortical localisation in the gibbon.

His first noteworthy independent research was upon the spinal cord of the monkey, in which he studied the immediate and remote effects of hemisection. In this investigation, which was published in the *Philosophical Transactions* for 1893, he obtained results not altogether in consonance with the views on conduction of sensory impressions in the cord which were at that time prevalent; but his observations have nevertheless proved to be accurate. This work established his position as a physiologist, and while it was still in progress he was appointed to the Assistant Professorship of Physiology in Liverpool, whence in the following year he again transferred to London to take up the Lectureship in the same subject at the Charing Cross Hospital Medical School.

As was at that time customary in most medical schools, he there combined the teaching of Physiology, and subsequently of Pathology, with clinical work, being eventually appointed Assistant Physician to the Hospital, and subsequently becoming full Physician and Lecturer on Medicine. The opportunities thus afforded for the scientific study of disease in general, and of the nervous system in particular, were taken advantage of by Mott with such success that when the London County Council in 1895 decided to appoint a pathologist



F. W. Mott

in charge of the laboratory they were proposing to establish at Claybury, all indications pointed to him as the most suitable occupant of the post. He, however, made the stipulation that he should not give up his clinical appointment at Charing Cross Hospital; considering it a matter of importance that the study of diseased conditions should be combined with opportunity for clinical observation. After some demur the Council gave way, and were thus able to secure Mott's services, which were rendered whole-heartedly for a long period of years, during which time a vast amount of work was carried out by him and his pupils and assistants. Most of this is published in a series of important volumes to which the title "Archives of Neurology" was given.

Subsequently, when the Maudsley Hospital for Mental Diseases was established at Denmark Hill, Mott's pathological work for the Council was transferred to the newer and better-equipped laboratory attached to that institution, and was continued there until his final resignation from the post of pathologist to the London County Council Asylums in 1923, the age limit having been stretched in order that his services might be continued as long as possible. But his activities were by no means at an end, for he continued to teach at the Maudsley Hospital, and also accepted the post of Lecturer on Morbid Psychology at the University of Birmingham. This position he held up to the time of his death.

Largely owing to Mott's researches it became definitely established that the hitherto obscure disease of the nervous system known under the designation "general paralysis of the insane," is, in fact, a manifestation of syphilis, and is associated with the presence of the specific spirochæte in the tissues of the central nervous system, and especially of the brain. The determination of the association between syphilitic infection and this and other mental disorders is the achievement by which Mott's name will probably be best known. But it was by no means his only important contribution to neuro-pathology, for he further demonstrated the close relation between the nervous system and the sexual organs which accompanies dementia præcox, as well as those associated with the deficient mental condition accompanying absence or degeneration of the thyroid.

Relying on the results of these researches, Mott firmly and insistently supported the view—which had been formulated many years before by Henry Maudsley, but had been strangely ignored—that mental disorders are, for the most part, correlated with bodily changes. Indeed, all his work tended to uphold this doctrine, which may be said to have abolished the idea previously current as to the impossible obscurity of the causation of diseases of the mind. Even in the affection known as "asylum dysentery" the cause used to be referred to a hypothetical nervous affection of the intestine, instead of to infection associated with micro-organisms, which was indicated by Mott to be its true origin. And as a result of his recommendations for isolation and disinfection, the disease, previously frequently fatal, has become either strikingly diminished or has ceased to appear.

On the outbreak of war Mott's services were secured by the Government, and he received a commission first as Major and afterwards as Lieutenant-Colonel in the Royal Army Medical Corps. In this capacity he was given charge of neurological cases at the IVth London General Hospital (King's College Hospital) and at the Maudsley Hospital, which was attached to it. Here he devoted himself to the investigation of the pathology and treatment of nervous conditions which were produced under the abnormal circumstances then prevalent, and in particular investigated the condition known as "shell shock"; this he showed to be accompanied in certain cases by actual physical changes in the central nervous system, although in other cases nothing of the kind could be observed. In recognition of his war services, as well as of his professional eminence, he was created K.B.E. (military side) in 1919. In the same year he received the Honorary Degree of LL.D. from the University of Edinburgh. A Fellow of the Royal College of Physicians of London since 1892, he gave successively the Croonian Lectures on the neurone doctrine, the Oliver-Sharpey Lectures on the cerebrospinal fluid, and the Lettsomian Lectures on the effect of high explosives on the nervous system; and by the Royal College of Physicians of Edinburgh he was invited to give the Morison Lectures, choosing for his subject "dementia præcox." He held for a time the Fullerian Professorship of Physiology in the Royal Institution. Only last year he delivered the Harveian Oration before the Royal College of Physicians of London. In this he dealt in a masterly fashion with heredity in relation to Mental Disease. At the time of his death he was President of the Royal Medico-Psychological Association, and was honorary member of many learned societies throughout the world.

Of a kindly disposition and always ready to assist other and younger men, he attracted a large number of workers, both from this country and from abroad, to the laboratories at Claybury and Denmark Hill, over which he presided. He enjoyed the esteem and affection of all who came in contact with him. He was transparently honest. Although holding strong opinions, he was always ready to jettison them if the evidence placed before him failed to justify them. For music he had a keen ear and a cultured taste. It was a source of much gratification to him that one of his daughters took up the serious study of that subject, and became a highly proficient 'cellist. One of the most notable features of his character was a strenuous loyalty—to his country, to his profession, to his colleagues, and to his friends. Few men will be more missed.

E. S.-S.

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